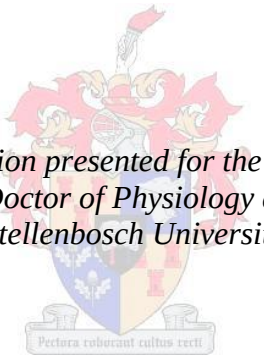
"The contribution of inflammatory mediators to delayed secondary muscle damage"

by

Mari van de Vyver



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Declaration

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ABSTRACT:

Background: Understanding the contribution of divergent individual response patterns remains a key objective in identifying mechanisms of inflammation and potential factors limiting the resolution of inflammation. The purpose of this research project was to investigate downstream effects of inflammation following exercise-induced muscle damage in human subjects.

Methods: For three different studies, a total of 53 untrained healthy male participants were recruited and divided into a non-exercising control (n=13) and exercise-induced muscle damage groups (n=40). The study design for the three studies was the same (with few exceptions): Downhill running (DHR) (12 x 5min bouts, 10% decline, 15 $km.h^{-1}$) with blood samples taken pre, post, after 2 and 4 hours post-exercise (2h, 4h) and on days 1, 2, 3, 4 and 7 (d1-d7). Serum was analysed for creatine kinase activity (CK), myoglobin (Mb), cortisol, cytokine (TNFα, IL-1ra, IL-1β, IL-4, IL-6, IL-8, IL-10, sIL-6R), chemokine (G-CSF, MIP-1β) and adhesion factor (sICAM-1, sP-selectin) concentrations. Tissue degradation was assessed by serum matrix metalloprotease (MMP-9) and myeloperoxidase (MPO) content. White blood cell differential count was determined and the surface expression of various cluster of differentiation factors (CD11b, CD163, CD68, CD88, CD34) as well as intracellular MPO were assessed in whole bood using flow cytometry. Nuclear localization of the inflammatory mediator NFκB in isolated perhipheral blood mononuclear cells (PBMCs) was determined using immunofluorescence microscopy. Muscle biopsies (*vastus lateralis*) taken at baseline, 4h, d1 and d2 were analysed for fibre type, inflammatory and stress-induced pathways (STAT3, IκBα, p38MAPK), myogenic factors (MyoD, myogenin), neutrophil activity (MPO) and satellite cell number (Pax7).

Results: Participants in the DHR group were subdivided into those with a normal recovery (DHR1) and those who developed secondary damage (DHR2). CK peaked on d1 in both subgroups (DHR1: 1512 ± 413 $u.L^{-1}$, DHR2: 1434 ± 202 $u.L^{-1}$) and again on d4 only in the DHR2 group (1110 ± 184 $u.L^{-1}$). A similar IL-6 and IL-10 response was evident immediately post DHR in all individuals. Additional IL-6 was released in the DHR2 subgroup peaking at 4h (10.3 ± 4.2 $pg.mL^{-1}$) whereas IL-10 had returned to baseline. IL-1ra (23.6 ± 8.8 $pg.mL^{-1}$), $CD68^{+}$ (5%) and $CD163^{+}$ (3%) monocytes were significantly higher in the DHR2 subgroup. Neutrophil count at 2h (DHR1: 8.6 ± 0.8 $x10^{9}$ $cells.L^{-1}$, DHR2: 11.4 ± 1.8 $x10^{9}$ $cells.L^{-1}$) was significantly ($p<0.02$) correlated to CK activity on d4. PBMC NFκB p65 nuclear localization

was slightly less at 2h in the DHR2 compared to the DHR1 and control groups. Intramuscular STAT3 signalling and MPO were significantly higher in the DHR2 compared to the DHR1 subgroup at 4h and d2 respectively. The progenitor cell response was similar for all DHR individuals with an increase in $Pax7^+$ SC observed at 4h (0.06 ± 0.01 Pax^+ SCs/fibre) and d1 (0.07 ± 0.02 Pax^+ SCs/fibre).

Conclusion: Healthy young men can be divided into those with a adequate and those with a less efficient capacity to control the post damage inflammatory response. The early cytokine response, especially IL-6, seems to be a key role player in the cascade of events leading to late secondary skeletal muscle damage.

OPSOMMING:

Agtergrond: Die begrip van uiteenlopende individuele reaksie patrone, is belangrik in die identifisering van faktore asook meganismes betrokke in die ontwikkeling en resolusie van inflammasie. Die doel van hierdie navorsingsprojek was om die gevolge van oefening-geïnduseerde spierskade en inflammasie te ondersoek in menslike proefpersone.

Metodiek: ‘n Totaal van 53 gesonde mans is tydens drie verskillende studies, gegroepeer in ’n kontrole (geen oefening) (n=13) en oefening geinduseerde spier skade (DHR) groep (n=40). Die uitleg van de studies was eenders (met min uitsonderings): Afdraende hardloop (12 x 5min hardloop sessies, 10% afdraende, 15km.h^{-1}) met bloed monsters geneem voor, na, 2 ure, 4 ure (pre, post, 2h, 4h) en op dag 1, 2, 3, 4 en 7 (d1-7). Serum is ontleed vir die volgende: kreatien kinase aktiwiteit (CK), kortisol, sitokiene (TNFα, IL-1ra, IL-1β, IL-4, IL-6, IL-8, IL-10, sIL-6R), chemokien (G-CSF, MIP-1β) en adhesie molekuul (sICAM-1, sP-selectin) konsentrasies. Weefsel degradasie is vasgestel deur die teenwoordigheid van matriks metalo-protease-9 (MMP-9) en miëloperoksidase (MPO) in serum te meet. Differensiële witbloed sel (WBC) telling asook die teenwoordigheid van sekere differensiasie faktore (CD11b, CD163, CD68, CD88, CD34) op die sel oppervlak asook intrasellulêre MPO vlakke is bepaal deur gebruik te maak van vloeisitometrie. Die lokalisering van NFκB in die selkerne van geïsoleerde bloed mononukleêre selle (PBMC) is bepaal deur fluoriserende mikroskopie. Spierbiopsies (*vastus lateralis*) geneem tydens rus (basislyn), na 4h, en op d1 en d2 is ontleed vir veseltipe, inflammatoriese en stresverwante faktore (STAT3, IκBα, p38 MAPK), miogeniese faktore (myoD, myogenin), neutrofiel aktiwiteit (MPO) en aantal satelliet selle (Pax7).

Resultate: Deelnemers in die DHR-groep is onderverdeel in twee groepe. Persone wat normaalweg herstel het is saam gegroepeer (DHR1) en diegene wat sekondêre skade ontwikkel het is saam gegroepeer (DHR2). CK aktiwiteit in serum het hoogtepunte bereik op d1 in beide subgroepe (DHR1: 1512 ± 413 u.L^{-1}, DHR2: 1434 ± 202 u.L^{-1}) en weer op d4 in die DHR2 groep (1110 ± 184 u.L^{-1}). 'n Soortgelyke IL-6 en IL-10 reaksie is onmiddellik na oefening (in al die proefpersone) waargeneem. Addisionele IL-6 is vrygestel in die DHR2 subgroep en het ’n hoogtepunt bereik na 4h (10.3 ± 4.2 pg.mL^{-1}), terwyl IL-10 reeds teruggekeer het na rustende waardes. IL-1ra (23.6 ± 8.8 pg.mL^{-1}), CD68^{+} (5%) en CD163^{+} (3%) monosiete was aansienlik hoër in die DHR2 subgroep. Neutrofieltelling na 2h (DHR1: 8.6 ± 0.8 x10^{9}cells.L^{-1}, DHR2: 11.4 ± 1.8 x10^{9}cells.L^{-1}) het verband ($p < 0,02$) gehou met

CK-aktiwiteit op d4. In vergelyking met die DHR1 en kontrole groep was die lokalisering van NFκB p65 in PBMC selkerne na 2h effens minder in die DHR2 subgroep. STAT3- en MPO-vlakke in die spiere was aansienlik hoër in die DHR2 subgroep as in die DHR1 subgroep na 4h en op d2 onderskeidelik. Die spierherstel proses was eenders vir alle individue wat aan die oefening deelgeneem het; 'n toename in $Pax7^+$ Satelietselle (SC) is waargeneem na 4h (0.06 ± 0.01 Pax^+ SC/spiervesel) en op d1 (0.07 ± 0.02 Pax^+ SC/spiervesel).

Gevolgtrekking: Gesonde jong mans kan verdeel word in diegene met 'n bevoegde en diegene met 'n minder doeltreffende vermoë om oefenings-geïnduseerde spierskade en die inflammatoriese reaksie te beheer. Die sitokien-reaksie, veral IL-6, blyk om 'n belangrike rolspeler in die ontwikkeling van sekondêre skeletspierskade te wees.

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ABBREVIATIONS:

ACE - angiotensin I converting enzyme

ANOVA – analysis of variance

ATP – adenosine triphosphate

bFGF - basic fibroblast growth factor

BMI – body mass index

Ca^{2+} - calcium

CCL2 - chemokine ligand 2

CCR2 - chemokine receptor 2

CD – cluster of differentiation

CK – creatine kinase

CLRs – C-leptin like receptors

COPD - chronic obstructive pulmonary disease

COX-2 - cyclooxygenase-2

CRP – C- reactive protein

CV- coefficient of variance

d – day

DAMPS – damage associated molecular patterns

DHR – downhill run

DLK – delta-like homoloque

DNA – deoxyribonucleic acid

DOMS- delayed onset muscle soreness

E-C – excitation-contraction

ecc- eccentric

ECM – extracellular matrix

EDL – *extensor digitorum longus*

EIMD – exercise-induced muscle damage

ERK - extracellular signal-regulated kinase

ex - exercise

FA-1 – fetal antigen-1

FABP – fatty acid binding protein

FKN - fractalkine

GAGs - glycosaminoglycans

G-CSF – granulocyte colony stimulating factor

GM-CSF- granulocyte macrophage colony stimulating factor

GPCRs – G protein coupled receptors

GSH - glutathione

h – hour

HCT – haematocrit

HGB – haemoglobin

HGF – hepatocyte growth factor

HMGb1 – high-mobility group box1

HPC – haematopoietic progenitor cells

HS – heparin sulphate

HSP- heat shock proteins

Hsp72 – heat shock protein 72

HSPG's – heparin sulphate proteoglycans

ICAM-1 – intracellular adhesion molecule-1

IFNα – Interferon alpha

IFNγ – Interferon gamma

IGF - insulin-like growth factors

IL-1ra – interleukin-1 receptor antagonist

IL-1β – interleukin-1β

IL-4 – interleukin-4

IL-6 – interleukin-6

IL-8 – interleukin-8

IL-10 – interleukin-10

IL-13 – interleukin-13

IL-17 – interleukin-17

iNOS – inducible nitric oxide

JAK - Janus kinases

JNK - Janus kinases

kg – kilogram

$kg.m^{-2}$ – kilogram per square meter

km - kilometer

$km.h^{-1}$ – kilometre per hour

$ml.min.kg^{-1}$ – millilitre per minute per kilogram

LDH – lactate dehydrogenase

LDLs – low density lipoproteins

LIF - leukaemia inhibitory factor

LPS - lipopolysaccharide

m - meter

MAPK - mitogen activated protein kinase

Mb – myoglobin

MCH – mean corpuscular haemoglobin

MCHC – mean corpuscular haemoglobin concentration

MCP-1 – macrophage chemoattractant protein-1

M-CSF – macrophage colony stimulating factor

MCV – mean corpuscular volume

MDC – macrophage derived chemokines

MEF – mouse embryonic fibroblasts

MEF-2 - myogenic enhancer factor 2

MHC – myosin heavy chain

min – minutes

MIP-1α - macrophage inflammatory protein 1 alpha

MIP-1β - macrophage inflammatory protein 1 beta

$ml.kg.min^{-1}$ – millilitre per kilogram per minute

mm – millimetre

MMP-9 –matrix metalloprotease-9

MPO – myeloperoxidase

MPV – mean platelet volume

MRF-4- myogenic regulatory factor-4

MRF's – myogenic regulatory factors

mRNA – messenger ribonucleic acid

N_2 – liquid nitrogen

NADPH – nicotinamide adenine dinucleotide phosphate

NCAM – neural cell adhesion molecule

NFAT - nuclear factor of activated T-cells

NFκB – nuclear factor-kappa B

$ng.mL^{-1}$ – nanogram per millilitre

NO – nitric oxide

NSAID- non-steroidal anti-inflammatory drug

OSM - oncostatin M

PBMC's - peripheral blood mononuclear cells

PCNA – proliferating cell nuclear antigen

PCR – polymerase chain reaction

PDGF - platelet derived growth factor

PE - phycoerythrin

p- phosho

PI3K - phosphatidylinositol-3-kinase

$pg.mL^{-1}$ – pegagrams per millilitre

PLT - platelets

PMN – polymorphonucleur neutrophils

Pref – preadipocyte factor

PSGL-1 - P-selectin glycoprotein ligand-1

PTS – peak treadmill speed

RBC – red blood cell

RDW - red cell distribution width

RER – respiratory exchange ratio

rh - recombinant human

RHD – Rel homology domain

RIP – receptor interacting proteins

RM – repetition maximum

ROS – reactive oxygen species

RT-PCR – real time polymerase chain reaction

SC – satellite cells

SE – standard error

sIL-6R – soluble interleukin-6 receptor

siRNA's – silencing RNAs

SNP's - single nucleotide polymorphisms

SOCS - suppressor of cytokine signalling

SOD – superoxide dismutase

SST – serum separating tube

STAT – signal transducer and activator of transcription

sTNFR – soluble tumour necrosis factor receptor

Th2 – T helper 2

TNFα –tumour necrosis factor α

TLRs – toll-like receptors

TLR-2 – toll-like receptor-2

TRADD – adaptor proteins

TRAF – receptor associated factors

$u.L^{-1}$ – units per litre

uPAR - urokinase type plasminogen-activator receptor

VCAM-1 – vascular adhesion molecule -1

VEGF - vascular endothelial growth factor

WBC – white blood cell

yr - years

°C – degrees centigrade

ųm - micrometer

CHAPTER 1: GENERAL OUTLINE

The main focus of the research presented in this dissertation was to improve our understanding of the contribution of divergent individual response patterns to the acute phase inflammatory response following exercise-induced skeletal muscle damage. This project furthermore aimed to identify potential factors involved in delaying the resolution of inflammation that ultimately resulted in a second phase of skeletal muscle damage.

Despite numerous studies investigating the responsiveness of the human immune system following acute exercise, very few researchers focus on inter-individual responses. *Chapter 2* will review the literature regarding the mechanisms behind the acute inflammatory response to exercise-induced muscle damage (EIMD), the regenerative phase of skeletal muscle and the involvement of cytokines and transcription factors in the interaction between exercising skeletal muscle and immune cell activity. Throughout the literature review specific attention will be paid to studies with human participants, highlighting individual variability previously observed. It is still largely unknown why certain otherwise healthy individuals are prone to developing sustained inflammation following an acute insult. Although it is well known that an exaggerated inflammatory response can cause additional damage to healthy tissue in animal models, a definitive mechanism for secondary damage in a human model is still lacking.

The research was undertaken as three acute downhill running intervention studies in human volunteers. By using intermittent high intensity downhill running as model to induce skeletal muscle micro-damage, we were able to identify three different response patterns. Participants *(healthy young men)* were thus divided into subgroups accordingly as discussed in *chapter 5*. Briefly, a small percentage of all participants showed evidence of exaggerated muscle damage *(exaggerated and delayed peak in creatine kinase (CK) activity >3000 u.L^{-1})* as evident in the appearance of indirect markers of muscle damage in circulation with a delayed peak observed on day 4. Data from these participants were however excluded from analysis since this exaggerated response can be associated with some genetic factors or an underlying myopathy. An early peak in indirect markers of muscle damage was observed in all of the remaining participants, in about one third of these participants the damage associated markers gradually returned to normal during the recovery phase as expected *(DHR1 subgroup)*. Whereas the remaining two thirds of the participants all experienced secondary muscle damage *(DHR2 subgroup)* during the late recovery phase. These divergent responses

were observed in response to the same intervention protocol in three separately recruited groups of healthy young men.

In addition to identifying individual response patterns, this research project furthermore aimed to investigate the subsequently triggered acute inflammatory response. One aim was to investigate in more detail the contribution of the inflammatory response to a second phase of muscle damage. As mentioned before, we were able to demonstrate that young healthy individuals, exposed to muscle micro-damage from unaccustomed eccentric exercise can be divided into those with a adequate and those with a less efficient capacity to control post-damage inflammatory processes. The observations leading to this conclusion are presented and discussed in *chapter 5*, together with evidence suggesting that the additional production of the cytokine, interleukin-6 (IL-6) might be involved in triggering a sustained inflammatory response in the subset of individuals who experienced secondary skeletal muscle damage. Downstream of IL-6, the intramuscular STAT3 signalling was significantly different between the two subgroups.

A second aim was to identify why certain individuals are predisposed to developing sustained inflammation. The final study therefore investigated the anti-inflammatory response to downhill running and the functional activity of mobilized immune cells *(circulating neutrophils and peripheral blood mononuclear cells*).

Chapter 6 demonstrates that despite a significant anti-inflammatory response occurring almost immediately following EIMD, the release of additional IL-6 into circulation occur after the resolution of the anti-inflammatory response. Furthermore, evidence are provided that implicate immune cell activity in the development of a second phase of skeletal muscle damage.

The main findings presented in this dissertation are summarized and discussed in *chapter 7*.

CHAPTER 2: LITERATURE REVIEW

The function of the immune system goes far beyond the host-defence responses. It is central to acute and chronic inflammatory conditions. The presence of low grade systemic inflammation may also dysregulate the normal acute inflammatory response. Either a low or an exaggerated inflammatory response would be undesired. The interaction between exercise and the immune system makes it an ideal model to study immune system responsiveness without the presence of any pathogens. In the case of tissue injury, the objective of inflammation is to prepare the injured tissue for regeneration so that function can be restored (Tidball JG 1995,2005). Dysregulation however poses an immune-derived risk for secondary damage to intact tissue adjacent to the injury site (*reviewed by* Cooper *et al.* 2007).

Although chronic inflammation is easily assessed using routine chemistry, it is not clear how to determine a propensity to develop sustained inflammation in otherwise healthy individuals. Intra-individual variability in immune responses are evident in all human studies as illustrated by the large standard deviations observed in comparison to animal models and/or *in vitro* studies. This suggests that humans respond differently given the same environmental or endogenous stimulus.

Early studies investigating the interaction between the immune system and exercise have been scrutinized in detail before, with some reviewers concluding that evidence has been mostly circumstantial and that various methodological discrepancies are present in the literature (Malm *et al.* 2001, St Pierre Schneider *et al.* 2007). Despite researchers focussing their investigations on specific aspects of the immune response to exercise in recent years, there is still a lack of understanding. Chemical mediators *(cytokines)* have both metabolic and immune functions and even opposing functions at different stages during recovery from tissue damage. Understanding the contribution of divergent individual response patterns remains a key objective in identifying mechanisms of inflammation and potential factors limiting the resolution of inflammation.

This chapter will review the literature regarding the mechanisms behind the acute inflammatory response to exercise-induced muscle damage (EIMD), including the mobilization of leukocytes *(refer to section 2.1.1, p. 23)*, their activation, adhesion to the vascular endothelium and transmigration to the injury site *(refer to section 2.1.2, p. 29)*. Specific attention will be given to studies undertaken with human subjects.

The intramuscular presence and activity of leukocytes following an acute bout of muscle damaging exercise in humans remain controversial *(refer to section 2.1.3, p. 33)* and the potential influence of methodological variables on results and conclusions will also be discussed. The focus will then shift to the functional activity of leukocytes in response to muscle damaging exercise and their contribution to a second phase of damage or promotion of regeneration *(refer to section 2.1.4, p. 36)*. This will be followed by an overview of skeletal muscle regeneration *(refer to section 2.2, p. 42)* within the context of EIMD as model.

Finally, despite the multiple signalling pathways activated in exercising skeletal muscle, the nuclear factor- kappaB (NFκB) signalling pathway is seen as the "master controller" of inflammation. Its activity in the *in vivo* context of exercise will therefore be discussed in detail *(refer to section 2.3, p. 54)*. The downstream targets of NFκB signalling *(such as Interleukin-6 (IL-6) up regulation)* could have modulating effects on both the inflammatory and regenerative responses. The final section of this chapter will therefore focus on IL-6 induced signal transducer and activator of transcription (STAT) signalling *(refer to section 2.4, p. 60)*.

2.1. Acute phase inflammatory response

2.1.1 Exercise-induced leukocytosis

Acute exercise *(with or without the presence of skeletal muscle damage)* is associated with the appearance of inflammatory mediators in circulation (Peake *et al.* 2008, Smith *et al.* 2000, 2007). The diverse functions of these inflammatory cytokines and chemokines are summarized in table 2.4 *(p. 40)*. From the table it is clear that many of these mediators play a role in leukocyte demargination, mobilization, activation, adhesion and/or migration (*reviewed by* Sanz *et al.* 2012, Smith *et al.* 2008, Williams *et al.* 2011). It is thus not surprising that an increase in total white blood cell (WBC) count *(leukocytosis)* is evident in most cases following an acute bout of exercise, even without the presence of severe skeletal muscle damage. It is important to note though that a large portion of human EIMD studies observing changes in inflammatory mediators failed to also investigate changes in WBC count (*refer to table 2.1, p. 27*). The increase in total WBC count is mainly driven by increases in neutrophils and monocytes. In comparison only minor changes occur in

lymphocyte, natural killer cell, eosinophil and basophil counts (Malm *et al.* 2000, Paulsen *et al.* 2005,2010).

A large number of immature neutrophils *(with bands)* (Suzuki *et al.* 1996, Yamada *et al.* 2002), are recruited from bone marrow and contribute to exercise-induced leukocytosis. During the process of hematopoiesis, immature cells *(undergoing granulopoiesis)* are known as band neutrophils or staff cells, with "band" referring to the shape of the nucleus. The mobilization of these leukocytes from the bone marrow in response to a variety of exercise stimuli, is at least in part mediated by IL-6, granulocyte colony stimulating factor (G-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF) (Paulsen *et al*. 2005, Subramaniyam *et al.* 2009, Suzuki *et al.* 2003,Yamada *et al.* 2002).

Elevated levels of circulating IL-6 in response to an acute exercise insult has been a consistent finding *(refer to table 2.1 p. 27)*. Studies that fit the following criteria: acute exercise intervention protocol *(not endurance type exercise)*; healthy young male subjects; measured at least 2 cytokines in circulation one of which had to be IL-6; subjects did not take any supplementation or received any form of treatment and the study did not investigate the effects of glucose metabolism, were included in Table 2.1 *(p. 27)*. Only 3 out of the 34 studies summarized in table 2.1 *(p. 27)* indicated no change in IL-6 concentrations following EIMD (Nosaka *et al.* 1996, Hirose *et al.* 2004, Uchida *et al.* 2009). From these studies, Nosaka *et al.* (1996) were the only one to also assess leukocytosis and were not able to detect any change in WBC count. The only other study that didn't observe any change in WBC count in response to EIMD (Hellsten *et al.* 1997) did however demonstrate a slight increase in circulating IL-6 concentrations.

In 2003, Natale *et al.* compared the effect of three different types of exercise on blood leukocyte count. A circuit of resistance exercise *(3x10 repetitions at 70% 1RM)* resulted in the largest changes in markers of muscle damage *(creatine kinase (CK) and delayed onset muscle soreness (DOMS)),* but prolonged aerobic exercise *(cycling at 60% VO_2max for 2 hours)* yielded the highest increases in total leukocytes and lymphocyte subsets *(CD3+(total T-lymphocyte), CD8+(cytotoxic and regulatory/ suppressor T-lymphocytes), CD3-CD16+/56+(natural killer cells))*.

In part, this may be related to the contribution of cellular stress and catecholamine release during prolonged aerobic exercise. In animals, catecholamines are known to play a role in the regulation of lipopolysaccharide (LPS)-induced neutrophilia through activation of alpha-

1-adrenoreceptors (Altenburg *et al.* 1997). Infusion of epinephrine *(30 minutes (min))* into healthy men also resulted in the demargination of leukocytes from the epithelium (Dimitrov *et al.* 2010). These studies did not include exercise. Nonetheless, even with exercise, systemic stress has been demonstrated to have a greater influence on leukocyte functional responses than the degree of muscle damage induced (Saxton *et al.* 2003). Using comparable eccentric exercise models that induce either low *(50 eccentric contractions with electrical stimulation superimposed)* or high *(40 min bench stepping)* levels of systemic stress, the authors investigated leukocyte phagocytic activity, respiratory burst and activation antigen (*CD11b, CD66b and CD64*) expression in healthy young men. Significant elevations in CK were only evident following eccentric contractions, but both models induced a similar increase in WBC count at 4 hours. Only the bench stepping exercise did however increase neutrophil and monocyte respiratory burst and phagocytic activity at 24 hours. The presentation of individual data demonstrate large variability in specifically neutrophil phagocytic activity following the bench stepping exercise. Definitive conclusions on the influence of systemic stress on leukocyte functional activity can however not be made since the level of systemic stress experienced by the individuals were not assessed.

In order to negate as much as possible the hormonal stress response to exercise, an exercise model involving eccentric contractions *(quadriceps)* at a low metabolic cost *(30% VO_2max)* was selected by Paulsen *et al.* (2005) to investigate the relationship between EIMD and leukocytosis. Despite the low metabolic cost of the exercise protocol, serum cortisol concentrations were elevated immediately post exercise. These changes were however unrelated to any change in circulating leukocyte number. An association was observed between the loss of muscle force, plasma CK activity, circulating leukocyte counts and C-reactive protein (CRP) concentrations (Paulsen *et al.* 2005). The authors therefore concluded that local events in the damaged muscle regulate the non-specific immune response to exercise and that the degree of muscle damage is reflected in the magnitude of leukocytosis. It remains difficult to distinguish between the metabolic effect of exercise on leukocytosis and an influence of tissue damage, especially at high intensities.

Within 24 hours following acute exercise, circulating numbers of leukocytes return to pre exercise levels. This can be as result of various factors, including spontaneous apoptosis (Park *et al.* 2011, Syu *et al.* 2011) or accumulation of these cells in injured tissue (Paulsen *et al.* 2010, Tidball *et al.* 1995, 2005). Park *et al.* (2011) investigated the effect of EIMD on the induction of apoptosis in peripheral blood leukocytes in healthy young men. Apoptotic

WBC's were evident up to 2 hours following level and downhill treadmill running *(40 min @ 70% VO_2max)*. A greater increase in leukocyte Bax concentration *(protein that promotes apoptosis)* and the Bax/Bcl-2 ratio *(Bcl-2 protects against oxidative stress and apoptosis)* were however observed at 24 and 48 hours following downhill running compared to level or a second downhill run. Based on this differential response the authors suggested that EIMD may be involved in the prolonged induction of leukocyte apoptosis.

Exercise-induced oxidative stress may be the mechanism for apoptosis upstream of the signalling proteins mentioned earlier. Increased neutrophil cytosolic and mitochondrial reactive oxygen species (ROS) production and glutathione *(antioxidant)* oxidation coincided with *ex vivo* Annexin-V binding on the outer surface of neutrophils *(indicative of apoptosis)* in response to acute severe exercise *(incremental cycling until exhaustion*) (Syu *et al.* 2011). In agreement, *in vitro* experiments show that exogenously added oxidants accelerate neutrophil apoptosis (Rollet-Labelle *et al.* 1998) whereas antioxidants inhibit apoptosis (Oishi *et al.* 1997). Suzuki *et al.* (1996) demonstrated that enhanced ROS generation by neutrophils *(measured by luminal-dependent chemiluminescence upon stimulation with opsonised zymosan)* following endurance exercise was associated with a proportional increase in staff cells. Together these studies suggest that despite mobilization of neutrophils, immature neutrophils may possess higher responsiveness and are likely to undergo apoptosis unless recruited into the damaged areas.

Table 2.1 Circulating inflammatory cytokines observed following acute exercise in human subjects.

First Author, date	Intervention protocol	Subject	time points	Inflammatory mediators measured		
				leukocytosis	increased from baseline	no change / undetectable
Concentric exercise						
Bruunsgaard 1997	Cycling 30min @65% VO_2max	healthy young male	20min, 30min, 2h, d2, d4, d7	Y	IL-6	IL-1b, TNFα
Ostrowski 1998	2.5h treadmill @75% VO_2max	endurance athletes	pre, 30min, 60min, 90min,2h, 3h, 4h, 5h, 6h, d2, d6	–	IL-6, IL-1ra	IL-8, IL-15, MIP-1a, MIP-1b
Suzuki 1999	90min cycling @ 90Watt for 3 days	untrained	pre, during, post, 1h, 3h, 12h	Y	IL-6, IL-8	IL1b, TNFα, IFNy
Yamada 2002	incremental treadmill ex to exhaustion	winter sports athletes	pre,post, 1h, 2h	Y	IL-6, G-CSF	
Vassilakopoulos 2003	45min cycling @70% VO_2max	untrained	pre, post, 30min, 2h	N	IL-6, IL1b, TNFα	
Conolly 2004	30 min cycle ergometer @ 80% VO_2max	healthy young male	pre, post, 1h	Y	IL-6, IL-1ra	
Sorichter 2006	60 min level treadmill @85% VO_2max	endurance athletes	pre, 1h,6h,d1,d3,d6	Y	Il-6,CRP	
Gokhale 2007	3 x 20 min running	athletes vs. non-athletes	pre, post, 1h	_	IL-6, TNFα	
Liang 2008	2h water emersion running @ 59% VO_2max	healthy young male	pre, post, 1h	Y	IL-6, G-CSF	
Peake 2008	90min cycling 65% VO_2max	trained cyclists	pre, post, 40min, 1.5h	Y	IL-6, IL-8, IL-10, IL-1ra, TNFα	
Eccentric exercise						
Nosaka 1996	24 max ecc rep elbow flexors	untrained	pre,post,d1,d2,d3,d4,d5	N		IL-1a, IL-1b, IL-6,TNFa, CRP
Bruunsgaard 1997	Ecc cycling 20min @100% VO_2max; 10min @ 150% VO_2max	healthy young male	20min, 30min, 2h, d2, d4, d7	Y	IL-6	
Hellsten 1997	5 bouts of one-legged ecc ex	healthy young male	pre, 45min, d1,d2,d4	N	IL-6	IL-1b
Brenner 1999	standard circuit training	healthy young male	for 5hours	Y	IL-6,IL-10,TNFa	
Croisier 1999	ecc isokinetic training	moderately active	pre, post	_	IL-6	
Smith 2000	ecc bench pres & leg curl 4 sets of 12 reps @ 100% RM	untrained	pre, 1.5h,6h,12h,d1,d2, d3,d4, d5, d6	_	IL-6, IL-10, M-CSF	TNFα, G-CSF, GM-CSF
Steensberg 2000	one legged knee extensions	untrained	pre, every h for 5hrs	_	IL-6	
Childs 2001	ecc contractions elbow	untrained	pre, post, d2,d3,d7	_	IL-6	

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MacIntyre 2001	quadriceps ecc ex 300 reps	Recreationally fit	pre,post, 2h, 4h, 6h, d1,d2,d3,d6,d9	Y	IL-6	
Steensberg 2002	knee-extensor ex 180min	healthy young male	pre, 30min, 90min, 180min	–	IL-6	TNFα
Toft 2002	60 min lower limb ecc ex	healthy young male	pre,post,4h,d5	_	IL-6	
Phillips 2003	ecc arm curl	healthy young male	pre,d7,d10,d14	_	IL-6, CRP	
Risoy 2003	60 min strength training in legs	well trained athletes	pre, post, 5min, 20min,35min, 50min, 65min, 5h,23h	Y	IL-6, G-CSF	IL-8, MIP-1b
Willoughby 2003	7 sets of 10 reps knee-extensor @150% 1RM	untrained	pre,post, 2h,4h, 6h,d1, d2, d4	–	IL-6	
Hirose 2004	elbow flexors dumbbell 90% flexed 6 sets of 5 reps	untrained	pre,post, 1h,3h,6h, d1, d2,d3, d4	–	G-CSF	IL-1b,IL-1ra, IL4, TNFα, IL-6, IL-10, IL12p40
Nieman 2004	weight lifting (4sets of 10 reps - 10 exercises)	strength trained	pre,post	Y	IL-6,IL-10, IL-1ra, IL-8	
Paulsen 2005	quadriceps ecc ex 300 reps	healthy young male	pre,post, 5h, 10h, 15h,20h,25h	Y	G-CSF,IL-6, MCP-1,M-CSF	IL-8, MIP-1b
Peake 2006	10 sets of 6 ecc contractions elbows	healthy young male	pre, post,1h,3h,d1 ,d2, d3	Y	IL-6	IL-10,IL-1ra, G-CSF, CRP
Miles 2008	elbow flexors 3 sets of 15 reps@ 1RM	untrained	pre, post, 4h, 8h, 12h, 16h, 20h, d1,d2	_	IL-6, sTNFR	
Uchida 2009	bench press different intensities	army soldiers	pre,post	_		IL-1b, IL-6, TNFα
Robson-Ansley 2010	6 sets of 10 reps knee flexion	Recreationally fit	pre, d1,d2,d3	_	IL-6, sIL-6R	
Ross 2010	5 sets of 10 unilateral leg press	healthy young male	pre,post,30min,1 h, 1.5h, 2h, 2.5h	–		IL-8, MCP-1, TNFα
Toth 2011	300 ecc contractions (quadriceps)	healthy young male	pre, 1h, 3h, d1	_	IL-6	
Downhill running						
Pyne 2000	DHR 40min @ 52% VO_2max	Well trained runners	pre,post,1h,6h,d 1	Y	Elastase	
Petersen 2001	5% DHR 90min @ 75% VO_2max	Recreationally fit	Pre,Post,d7,d14	Y	IL-6, IL-1ra	
Thompson 2004	18% DHR 30min	healthy young male	up to 3d	_	IL-6	
Peake 2005	10% DHR 45min 65% VO_2max	well trained triathletes	pre, post, 1h, d1	Y	IL-6	IL-8, G-CSF
Peake 2005	10% DHR 45min 65% VO_2max	well trained runners	pre,post,1h,d1	_	IL-1ra, IL12p40, MCP-1,IL-10	IL-4

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Kingsley 2006	17.5% DHR 30min @ 70% HR	Untrained	pre, post, d1,d2	_	IL-6	
Smith 2007	13.5% DHR 60 min	Untrained	pre,post, hourly for 12h,daily for 6d	–	IL-6, IL-10, IL12(p70),IL-7,IL-8, MCP-1, MIP-1b	IL-4, IL-2, IFNy,IL-5,IL-13, IL-17, IL-1b, TNFa,G-CSF, GM-CSF
Buford 2009	17.5% DHR 45min @60% VO_2max	active young men	pre, 3h, d1	–	IL-6,IL-8, IL-12, TNFα	
Broadbent 2010	40 min DHR	Recreationally fit	pre,post,d1, d5	Y	IL-6	
Zembron-Lacny 2010	90 min @65% VO_2max final 15min DHR	Recreationally fit	pre,20min ,d1,d2	–	IL-6, IL-10, IL-1b, TNFα	
Pumpa 2011	10% DHR 5 bouts x 8min 80%HR	well trained	pre,post, 4h,d1,d2,d3 ,d4	–	IL-6, IL-10	IL-1, TNFα
van de Vyver 2012	10% DHR 12x5min @ 75%VO_2max	Untrained	pre,post,d1,d2,d3, d4, d5, d6,d7,d8, d9	–	IL-6,IL-10, TNFα	

Footnote: This table include all human studies with healthy young male subjects that measured circulating cytokines/chemokines following an acute bout of exercise. Abbr: CRP – C reactive protein, DHR – downhill run, ecc – eccentric, ex – exercise, G-CSF – granulocyte colony stimulating factor, h – hour, IFN – interferon, IL – interleukin, MCP – macrophage chemotractant protein, M-CSF – macrophage colony stimulating factor, MIP – macrophage inflammatory protein, min – minutes, N – no, sTNFR – soluble tumour necrosis factor alpha receptor, sIL-6R – soluble interleukin-6 receptor, TNF – tumour necrosis factor.

2.1.2 Leukocyte activation to trans-endothelial migration

Once mobilized, circulating leukocytes are activated and express heparin sulphate proteoglycans (HSPG's) that interact with activated vascular endothelial cells (Djanani *et al.* 2006, Dunzendorfer *et al.* 2001, van der Voort *et al.* 2000) causing the leukocytes to adhere and crawl along the endothelial surface (*reviewed by* Sanz *et al.* 2012, Smith *et al.* 2008, Williams *et al*. 2011).

In addition to these HSPG's, the surface expression of other receptors is commonly used as indication of leukocyte activation status (*refer to table 2.2, p. 30*). Many of these markers are however non-specific and expressed on more than one cell type, making it difficult to distinguish between the functional status of different leukocytes.

Table 2.2 Leukocyte *(macrophage and neutrophil)* activation markers

Antigen	Expressed on	Function	First Author, date
CD4	T-helper cells; monocytes	accessory molecule for TCR; antigen recognition; MHCII interaction	Litman 1987
CD11b (Mac1;CD18;αM β integrin)	NK cells; T cells; MP; Neu	mediates adhesion to endothelium; receptor for c3b1	Tan 2012
CD14	monocytes	LPS binding protein; activation marker; induce oxidative burst	Stelter 2000
CD15	Granulocytes	mediates phagocytosis and chemotaxis	Nakayama 2001
CD16	NK cells; monocytes; Neu	low affinity Fc receptorfor IgG	Vivier 1992
CD23	B-cells; monocytes	Trigger monokine release; IgE synthesis; pro-inflammatory	Bonnefoy 1997
CD45	All leukocytes	cytoplasmic phosphatase activity; signal transduction; apoptosis	Towbridge 1994
CD54	All leukocytes	ICAM-1, adhesion molecule	Ybarrondo 2001
CD62L (selectins)	B-cells; T-cells; NK cells; monocytes; Granulocytes	Homing receptor on leukocytes	Bevilacqua 1993
CD68	MP	receptor for oxidized LDLs	Rabinowitz 1991
CD163	MP, monocytes	scavenger receptor	Onofre 2009
CD206	MP	C-type lectin receptor expressed during phagocytosis	wollenberg 2002

Footnote: Abbr: CD – cluster of differentiation, ICAN – intracellular adhesion molecule, LDLs – low density lipoproteins, LPS - lipopolysaccharide, MP – macrophages, Neu – neutrophils, NK – natural killer, TCR – T cell receptor.

Presence of circulating pro-inflammatory cytokines and conditions such as acute muscle damage are associated with an increase in leukocyte chemotactic activity (Okutsu *et al*. 2008, Ortega *et al.* 1999, Paulsen *et al.* 2005, Risoy *et al.* 2003, Syu *et al.* 2012). Paulsen *et al.* (2005) reported large inter-individual differences in chemotactic responsiveness following 300 eccentric contractions of the *quadriceps* muscle group and support our hypothesis that different humans respond differently given the same stimulus. However, the different underlying biological responses have not yet been established.

In acute tissue injury models, necrosis and/or increased permeability of the plasma membrane lead to the release of damage associated molecules *(which are usually confined intracellularly)* (*reviewed by* Chen *et al.* 2010, Williams *et al*. 2011). Damage-associated molecules derived from necrotic cells include chromatin-associated protein high-mobility group box1 (HMGb1) (Scaffidi *et al*. 2002) and heat shock proteins (HSPs) (Quintana *et al.* 2005). EIMD is known to be associated with an increase in serum heat shock protein 72 (Hsp72) (Fehrenbach *et al.* 2005, Ogawa *et al.* 2011, Walsh *et al*. 2001). Upon extracellular matrix degradation, fragments such as hyaluronan (Jiang *et al*. 2005, Scheibner *et al.* 2006, Taylor *et al.* 2007) and heparin sulphate (Johnson *et al*. 2002) are released and can also be considered damage associated molecules. An increase in serum hyaluronan concentration have previously been demonstrated during exercise *(quadriceps femoris*, *anterior tibial muscle groups)*(Piehl-Aulin *et al.* 1991). Despite muscle-damaging exercise rarely being associated with necrosis, sarcolemmal integrity is compromised in most cases. However, since the sarcolemma is relatively easily patched, molecules associated with necrosis are not consistently seen in circulation.

Based on animal model experiments it is known that damage associated molecules enhance the endothelial expression of chemokines that in turn play a role in the recruitment of leukocytes to the damaged area (Woodfin *et al.* 2010, Wong *et al.* 2010). Griffin *et al.* (2012) recently demonstrated that the pro-inflammatory cytokines, tumour necrosis factor α (TNFα), interleukin-1β (IL-1β), interleukin-17 (IL-17) and interferon-γ (IFNγ) also have the ability to induce the endothelial expression of chemokines *(CXCL1, CXCL2, CXCL5, CXCL9, CXCL10 and CCL5) in vitro*. The interaction between these chemokines and specific G-protein coupled receptors (GPCRs) expressed on the leukocyte surface play a role in leukocyte recruitment. These include toll-like receptors (TLRs) *(transmembrane proteins)*, C-type lectin receptors (CLRs) *(transmembrane proteins with a carbohydrate binding domain, usually involved in microbial defense)*, CXC receptors and CCL receptors (Iwasaki *et al*. 2010, Takeuchi *et al.* 2010, Williams *et al.* 2011).

Although the endothelial expression of chemokines is less well defined in humans, interleukin-8 (IL-8) and hsp72 are thought to be potent polymorphonuclear (PMN) chemoattractants (Ludwig *et al.* 1997, Ortega *et al.* 2009,2010, Schlorke *et al.* 2012). Ortega *et al.* (2009) demonstrated that physiological concentrations of Hsp72 observed following a single bout of exercise (*1h cycle ergometer @ 70% VO_2max*) in sedentary women stimulated neutrophil chemotaxis *in vitro* through its interaction with toll-like receptor-2 (TLR-2). The

same authors later on confirmed the role of TLR-2 and Hsp72 in neutrophil phagocytic activity post exercise (Giraldo *et al*. 2010). By stimulating human peripheral neutrophils *ex vivo* with concentrations equivalent to post-exercise serum Hsp72, the authors furthermore demonstrated that an increase in phagocytic capacity of neutrophils is mediated through intracellular phosphatidylinositol-3-kinase (PI3K), extracellular signal-regulated kinase (ERK) and NFκB signalling (Giraldo *et al*. 2010).

Activated leukocytes can thus produce additional pro-inflammatory cytokines in response to NFκB signalling and therefore enhance the acute phase response and participate in endothelial activation. Upon endothelial activation, endothelial storage granules fuse with the endothelial plasma membrane and contribute to the surface expression of P-selectin and/or E-selectin. These selectins then interact with their ligands expressed on leukocytes *(eg. P-selectin on endothelial surface bind to P-selectin glycoprotein ligand-1 (PSGL-1) on neutrophil surface (Kansas et al. 1996))* thereby slowing down the movement of circulating leukocytes. The slow moving neutrophils detect chemokines more easily. The chemokines are not released into circulation but are immobilized on the luminal membrane of endothelial cells by damage associated molecules such as heparin sulphate (HS) or glycosaminoglycans (GAGs) (Middleton *et al.* 2002, Handel *et al.* 2005, Wang *et al.* 2005). The detection of these immobilized chemokines by leukocytes result in the activation of leukocyte integrins (Evans *et al.* 2009, Johnson *et al*. 2005, Luo *et al.* 2007). Monocytes, lymphocytes, eosinophils and basophils all express α4β1 integrins that bind to vascular cell adhesion molecule (VCAM-1) (Berlin *et al.* 1995, Elices *et al.* 1990), whereas neutrophils express β2 integrin that binds to intracellular adhesion molecule-1 (ICAM-1) on the endothelial surface (Arnaout *et al.* 1990). Binding results in leukocyte adhesion to the endothelium.

In addition, the activation of leukocyte integrins cause a conformation change in leukocytes *(from inactive to fully extended with a high affinity for binding)* (Evans *et al.* 2009, Luo *et al.* 2007). Neutrophils for example flatten and extend pseudopods that allow the cell to crawl along the endothelial surface (Ley *et al.* 2007). Massena *et al.* (2010) illustrated that αMβ2 integrins *(also known as Mac-1, CD11b,CD18)* mediate leukocyte crawling along the endothelium. It also guides the leukocytes towards tricellular junctional regions for migration across the vascular endothelial monolayer (Phillipson *et al.* 2006, Sumagin *et al.* 2010). The transmigrating leukocytes cross the vascular basement membrane into the extracellular matrix (ECM) at areas containing low matrix protein deposits (Voisin *et al.* 2010). Once inside the ECM neutrophils move along the chemical gradient to the chemokine

source and injured area. In the case of EIMD it is possible that damage to the ECM leading to areas with less matrix than usual facilitates transmigration. There is some controversy regarding the intramuscular presence and activity of leukocytes following an acute bout of muscle damaging exercise.

2.1.3 Leukocyte infiltration

Exercise-related human studies which have assessed intramuscular leukocyte infiltration are presented in table 2.3 *(p. 35)*. In an attempt to clarify the relationship between neutrophil infiltration and exercise-induced muscle injury St Pierre Schneider *et al.* (2007) reviewed 38 human and animal studies published between 1950 and 2007 that focussed on mechanically induced muscle injury. The results from these studies were very diverse with 85% of the animal models and only 55% of the human studies showing signs of intramuscular neutrophil infiltration following EIMD.

In addition to the effect of the exercise protocol itself, concerns have been raised regarding the influence of the muscle biopsy procedure on neutrophil infiltration. These concerns are based on findings from a single study by Malm *et al.* (2000) that indicated significant neutrophil infiltration *(CD11b, CD15)* in both exercised and non-exercised muscles compared with baseline. However, the study by Malm *et al.* (2000) involved 7 biopsies *(3 or 4 per leg)* within a period of 7 days. To refute or confirm the conclusions studies should firstly have fewer biopsies per leg with a larger distance between biopsy sites. Secondly, a control group is essential to account for the effects that the muscle biopsy procedure might have. In addition to methodological variables, divergent individual responses could account for varying findings between studies, making it difficult to establish a true relationship between exercise-induced muscle damage and leukocyte activity.

In an innovative study, Paulsen *et al.* (2010) were able to determine the exact tissue localization and overall distribution of leukocytes in the human *quadriceps* muscle group in both an exercising and non-exercising control leg following 300 eccentric contractions. A combination of scintigraphic imaging of transfused autologous radiolabelled *(99m Technetium)* leukocytes and immunofluorescent microscopy *(CD16 and CD68)* of tissue samples were used to demonstrate early accumulation of leukocytes in muscle endomysium and perimysium following exercise. The authors further demonstrated that in a small number

of subjects *(high CK responders)*, large increases in CK were associated with a larger accumulation of radio-labelled leukocytes.

Paulsen *et al.* (2010) also observed additional CK appearance in circulation during the recovery phase *(day 4)* in these particular subjects. Van de Vyver *et al.* (2012) recently observed a similar biphasic response of three different indirect markers of muscle damage *(CK, myoglobin (Mb), lactate dehydrogenase (LDH))* following intermittent downhill running and ruled out the biopsy procedure as possible cause for the additional leakage of muscle proteins into circulation. The initial increase in markers of muscle damage coincided with elevated levels of pro-inflammatory IL-6 and TNFα suggesting a link between inflammation and secondary muscle damage (van de Vyver *et al.* 2012).

Despite overwhelming evidence from animal studies suggesting that neutrophils in injured muscle can act as cytotoxic agents with the potential to cause additional damage *(refer to section 2.1.4, p. 36)*, a definitive cause and effect relationship has not yet been established in humans. Paulsen *et al.* (2010) demonstrated that very few of the circulating neutrophils *(99m Technetium labelled, $CD16^{+}$)* captured in the intramuscular microvessels shortly after exercise, crossed the endothelium within the time frame of sampling in their study *(0.5h, 4h, 8h, 24h, 96h, 168h)*. An increased number of intramuscular macrophages during the later recovery phase *(day2 to day7)* following exercise-induced muscle damage has been a more consistent finding *(refer to table 2.3, p. 35).*

Interleukin-6 , and specifically soluble interleukin-6 receptor (sIL-6R) dynamics have been implicated in controlling the pattern of leukocyte recruitment following acute EIMD (Hurst *et al.* 2001, Robson-Ansley *et al.* 2010). Hurst *et al.* (2001) demonstrated that infiltrating neutrophils shed sIL-6R from their cell surfaces and that this receptor regulated neutrophil activating chemokines which in turn suppressed neutrophil recruitment and enhanced the attraction of monocytes. Robson-Ansley *et al.* (2010) observed a biphasic pattern with sIL-6R significantly decreased at 48 and 72 hours following eccentric exercise *(6 sets of 10 knee flexion)* and hypothesised that an increase in sIL-6R is associated with suppressed neutrophil and enhanced monocyte infiltration into the damaged tissue soon after EIMD whereas the later decrease in sIL-6R could be associated with suppressed macrophage infiltration and the start of regeneration. Although not directly assessed, there was an association between sIL-6R, perceived pain and reduced peak muscle performance (Robson-Ansley *et al.* 2010).

The initial appearance of markers of muscle damage in circulation is as result of mechanical damage to skeletal muscle induced by exercise, whereas a secondary increase during the late recovery phase is indicative of secondary skeletal muscle damage *(unrelated to the mechanical insult).* This secondary increase in markers of muscle damage, occur one or two days following peak neutrophil activity and coincides with an increase in intramuscular macrophage activity.

Table 2.3 Intramuscular presence of leukocytes following acute muscle damaging exercise.

First Author, date	Intervention protocol	Detection	Neutrophils *(peak)*	Macrophages *(peak)*	Leukocyte
Hikida 1983	Marathon	EM			day1
Jones 1986	eccentric contractions calves, forearm	H&E		day7	
O'Reilly 1987	45min eccentric cycling	EM			day10
Round 1987	eccentric stretch injury	EM		days 9-14	
Fielding 1993	45min 16% downhill @70%VO_2max	Methylene blue	45min, day5		
MacIntyre 1996	300 maximal isokinetic eccentric contractions quadriceps	Tc99m-labelled	day1		
Hellsten 1997	maximal eccentric contractions quadriceps	CD11b	day2		
Bourgeois 1999	6 sets of 10 reps leg press @ 80%	H&E CD45			No change No change
Child 1999	70 maximal ecc knee extentions	H&E			day7
MacIntyre 2000	300 maximal isokinetic eccentric contractions quadriceps	Tc99m-labelled	2h,4h		
Malm 2000	incremental cycling (100W 60rev.min-1; increase 50W /2min)	CD11b CD15 CD163	No change 4h	 days 2,4	
O'Grady 2000	20 min stepping	H&E			post
Stupka 2001	eccentric knee extentions	MPO CD68	No change	 day1	
MacIntyre 2001	300 maximal isokinetic eccentric contractions quadriceps	Tc99m-labelled	6h		
Beaton 2002	240 maximal isokinetic eccentric contractions quadriceps	MPO CD68	No change	 day1	
Beaton 2002	300 maximal isokinetic eccentric contractions quadriceps	MPO Elastase CD68	day1 day1	 4h, day1	

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Raastad 2003	leg press (5 sets of 3 max reps)	Tc99m-labelled	20h		
Peterson 2003	eccentric knee extentions (14sets of 10 reps, 120% 1RM)	CD15	No change		
		CD68		day1	
Malm 2004	45 min treadmill (4% uphill, or 4 or 8% downhill)	CD11b	No change		
Dennis 2004	Resistance exercise circuit	CD68		day3	
Crameri 2004	eccentric knee extentions (3x 8 sets of 10 reps)	CD68		days 2,4,8	
Przbyla 2006	Resistance exercise circuit	CD68		No change	
		CD11b		day3	
		CD163		day3	
Hubal 2008	300 eccentric quadricep contractions	Mac387		No change	
Lauritzen 2009	70 unilateral max ecc contractions elbow	EM			days 4,7
Mikkelsen 2009	200 eccentric quadricep	CD16	No change		
		CD68		No change	
Paulsen 2010	14sets of 5 reps unilateral ecc contractions elbow	Tc99m-labelled	20h	days 2,4,7	
		CD68			
Paulsen 2010	300 eccentric contractions qaudriceps	CD16	day4		
		CD68		day4	
MacNeil 2011	150 eccentric contractions qaudriceps	MPO	day2		
		CD68		day2	

Footnote: Table includes all human studies that investigated intramuscular presence of leukocytes in healthy young men following acute exercise. **Abbr:** CD – cluster of differentiation, d – day, ecc – eccentric, EM – electron microscopy, ex – exercise, h – hour, H&E – hematoxilyn and eosin, MPO – myeloperoxidase, reps – repetitions.

2.1.4 Neutrophils versus Macrophages: contribution to secondary damage or essential for repair

Neutrophil functional activities are usually accompanied by varying degrees of tissue destruction. *In vitro* stimulation of isolated polymorphonuclear cells with inflammatory mediators such as LPS, TNFα, IL-8 and G-CSF results in the release of large quantities of matrix metalloprotease-9 (MMP-9) (Pugin *et al*. 1999). MMP-9 is known to play a role in tissue destruction by degrading extracellular matrix components (Ohbayashi *et al.* 2002, Van den Steen *et al.* 2002). The binding of the chemokine IL-8 to its CXCR2 receptor is thought to be responsible for the release of MMP-9 from neutrophil granules (Chakrabarti *et al.* 2005).

Neutrophils also generate high quantities of superoxide *(nicotinamide adenine dinucleotide phosphate (NADPH) oxidase dependent reaction)* and hypochlorous acid *(myeloperoxidase (MPO) dependent reaction)* (*reviewed by* Toumi *et al.* 2006). These oxidizing reactions associated with the respiratory burst activity of neutrophils may also be responsible for collateral damage to uninjured tissue. Brickson *et al*. 2003 demonstrated that blocking neutrophil activation *(through M1/70 antibody against CD11b),* thereby preventing the subsequent respiratory burst, minimizes myofibre damage 24 hours following stretch injury in white rabbits.

In agreement, Nguyen *et al.* (2005) investigated activation and cytotoxic capacity of neutrophils and identified neutrophil-mediated lysis of muscle cells through an MPO dependent pathway following mechanical loading. The study demonstrated that neither cyclic loading of muscle (C2C12) cells *in vitro* nor the presence of superoxide dismutase (SOD) *(enzyme responsible for converting superoxide to hydrogen peroxide)* in neutrophil-C2C12 co-cultures alone resulted in cell lysis. In contrast, the application of cyclic mechanical loading to co-cultures in the presence of SOD activated neutrophil cytotoxic activity. No cell lysis was however observed in MPO null mutant neutrophil (MPO-/-) and C2C12 co-cultures following mechanical loading. This phenomenon was confirmed in an animal model where a significant reduction (52%) in membrane lysis were observed in MPO-/- compared to wild type mice in response to reloading after a period of unloading (Nguyen *et al.* 2005).

Increases in both MPO and MMP-9 *(indicative of neutrophil degranulation)* were recently shown to be associated with an increase in serum IL-6 from pre to immediately post exercise *(incremental cycling to exhaustion)* in professional male athletes (Reihmane *et al.* 2012). This association suggests that the pro-inflammatory cytokine response *(especially IL-6)* is likely to play a role in modulating post exercise changes in immune function with the potential to compromise sarcolemmal integrity.

Furthermore, MPO and/or pro-inflammatory cytokines *(IFNγ, TNFα)* result in the classical activation of macrophages *(pro-inflammatory phenotype, M1)* (Mackaness *et al.* 1964). These macrophages express a receptor for oxidized low-density lipoproteins (LDLs), CD68 *(also known as macrosialin, ED1 antigen)* (Ottnad *et al.* 1995, Rabinowitz *et al.* 1991). Oxidative modification of LDLs by MPO promote their binding to CD68 and as consequence activate macrophages to increase their production of pro-inflammatory cytokines (*reviewed by* Tidball

et al. 2010). In addition to amplifying inflammation, these pro-inflammatory macrophages have the ability to cause additional muscle damage through the production of cytotoxic nitric oxide (NO) *(inducible nitric oxide synthase (iNOS) converts arginine to citrulline and NO*) (Albina *et al.* 1990,Nguyen *et al.* 2003). Silveira *et al.* (2007) demonstrated in an animal model that one hour of swimming increased circulating monocyte/macrophage phagocytic activity and that the expression of iNOS was associated with the release of hydrogen peroxide *in vitro*. This cytolytic capacity of M1 macrophages is promoted in the presence of neutrophils and ultimately results in a positive feedback loop that promotes phagocytosis. Although phagocytosis can cause additional damage, it plays an important role in the scavenging process and removal of cellular debris in order to prepare the injured area for repair. Teixeira *et al.* (2003) demonstrated that mice depleted of neutrophils had large areas of necrotic tissue 7 days following snake venom injection whereas control animals showed successful regeneration at the same time point, suggesting that neutrophil-mediated phagocytosis plays a prominent role in skeletal muscle regeneration.

In addition to amplifying the inflammatory response *(through NFκB signalling*), the release of pro-inflammatory cytokines *(TNFα, IL-6)* have been shown to induce the proliferation of muscle specific progenitor cells *(satellite cells) in vitro* (Li *et al.* 2003). Chazaud *et al.* (2003) investigated the interaction between isolated human peripheral blood mononuclear and myogenic progenitor cells *(myoblasts)*. The authors used DNA micro array analysis together with RT-PCR and identified in myoblasts that increased gene expression of monocyte chemoattractant protein-1 (MCP-1, *also known as CCL2*), macrophage –derived chemokine (MDC, *also known as CCL22*), fractalkine (FKN, *also known as* CX_3CL1), vascular endothelial growth factor (VEGF) and urokinase type plasminogen-activator receptor (uPAR) promoted monocyte chemotaxis *in vitro*. In addition, they demonstrated that 35% of myoblasts *(in vitro C2C12 or primary SC)* die upon total growth factor deprivation, but in the presence of M1 macrophages these cells were protected against apoptosis.

Macrophages play another important role in regeneration. Macrophage phenotype switches from M1 to the anti-inflammatory M2 phenotype (Graff *et al.* 2012, Tidball *et al.* 2010, Villalta *et al.* 2009). Based on functional and molecular specialization (Mantovani *et al.* 2004) the M2 macrophages have been subdivided into M2a *(*$CD68^+CD206^+$*)(anti-inflammatory and tissue remodelling roles)* (Martinez *et al.* 2008), M2b *(Th2 differentiation)*(Anderson *et al.* 2002) and M2c *(*$CD68^+CD206^+CD163^+$*) (promoting repair)* (Villalta *et al.* 2009) subcategories. To date, investigations into the intramuscular presence

and activity of macrophages following acute muscle-damaging exercise in humans, have failed to distinguish between the different subtypes (*refer to table 2.3, p. 35*). From *table 2.3* it is clear that only Przbyla *et al.* (2006) investigated the presence of both pro- and anti-inflammatory macrophages in the human *vastus lateralis* muscle following EIMD *(3 x 8 repetitions of a circuit of leg resistance exercises)*. The data are difficult to interpret since no change in the total number of intramuscular macrophages ($CD68^+$) does not seem to be consistent with an increase in both $CD11b^+$ pro-inflammatory *(55% increase)* and $CD163^+$ anti-inflammatory macrophages *(29% increase)* at 72 hours post exercise. The authors concluded that a small percentage of resident macrophages contribute to inflammation whereas the rest have alternative functions. Macrophages in the process of undergoing phenotype switching may however co-express different activation markers.

In a comprehensive study using both C2C12/ macrophage co-culture and the *mdx* mouse model, Villalta *et al.* (2009) investigated the mechanism by which macrophages promote muscular dystrophy. The specific focus was on their phenotype during stages of peak muscle necrosis or regeneration. The authors confirmed that M1 macrophages *(activated by INFγ, TNFα)* contributed to muscle lysis *in vitro* through NO dependant mechanisms and that genetic ablation of iNOS significantly reduced membrane lysis *in vivo*. More importantly the authors were able to demonstrate that M2a macrophages *(activated by interleukin-4 (IL-4),interleukin-13 (IL-13))* decreased the *in vitro* membrane lysis of M1 macrophages through the expression of arginase. Arginase *(originating from M2a)* competes with iNOS *(originating from M1)* for binding to their common enzymatic substrate, arginine. An increase in the expression of IL-4 and interleukin-10 (IL-10) by M2a macrophages furthermore resulted in the deactivation of M1 macrophages *(reduced expression of iNOS, IL-6, MCP-1)* and activation of M2c macrophages *(expressing interleukin-1 receptor antagonist (IL-1ra), IL-4, IL-10)* during the regenerative phase (Villalta *et al.* 2009). The precise mechanism involved in phenotype switching is still unclear but it is hypothesized that it might involve the modulation of microRNA (Graff *et al.* 2012). The effect of microRNAs on target gene expression and translation may give macrophages the capacity to respond with different patterns of activation to various environmental stimuli.

Villalta *et al.* (2009) also demonstrated that *in vitro* IL-10 stimulated M2c macrophages mediated regeneration through promotion of satellite cell (C2C12) proliferation. IL-10 production and release is not the only anti-inflammatory mechanism. The inhibition of pro-inflammatory cytokine production also occurs through signal transducer and activator of

transcription / suppressor of cytokine signalling (STAT3/SOCS) signalling (Murray *et al.* 2006, Schroder *et al*. 2003).

In summary, it seems that despite their capacity to cause additional damage to healthy tissue, the phagocytic activity of both neutrophils and pro-inflammatory macrophages is essential in preparing the injured area for regeneration. Both macrophage subtypes play a role in enhancing the myogenic regeneration response by influencing satellite cell function *(refer to next section)* Nevertheless, although the acute inflammatory response seems to be essential for skeletal muscle regeneration, an exaggerated response or dysregulation thereof can delay regeneration and even result in a second phase of damage.

Table 2.4 Possible functions of circulating inflammatory mediators following an acute bout of exercise.

		Possible actions following acute exercise	First Author, date
Cytokine/ Chemokine			
G-CSF	Granulocyte colony stimulating factor	Leukocyte (granulocyte) mobilization	Yamada 2002
		↓ neutrophil apoptosis	Mooren 2012
		Progenitor cell mobilization	Huttman 2006
		↓ satellite cell apoptosis and ↑ proliferation in animal model	Stratos 2007
GM-CSF	Granulocyte macrophage colony stimulating factor	Leukocyte mobilization	Subramaniyam 2009
		Progenitor cell mobilization	Subramaniyam 2009
IFNα	Interferon alpha	Role in immune mediated fatigue in animal model	Davis 1998
IFNγ	Interferon gamma	Classical activation of marcophages; ↓ M2 macrophage activation	Villalta 2011
		Activates ICAM-1; ↑ neutrophil migration from circulation to injured tissue	Gao 1996
		↑ trauma-induced muscle wasting	Madihally 2002
		↑ Satellite cell proliferation	Olsson 1994; Villalta 2011
IL-1ra	Interleukin-1 receptor antagonist	Anti-inflammatory, block IL-1 receptors	Steensberg 2003

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IL-1β	Interleukin-1 beta	↓ IGF-1 secretion; ↑ protein turnover	Fan 1996
		↑ Endothelial secretion of G-CSF and M-CSF	Zsebo 1988
		↑ expression of ICAM-1 and VCAM-1	Cannon 1998
		↑ expression of IL-6 and MCP-1	Galluci 1998
		Satellite proliferation; inhibits differentiation	Allen 1989
IL-4	Interleukin-4	B cell and T cell proliferation	Paul 1987
		↑ alternative M2 activation of macrophages	Lyamina 2012
IL-5	Interleukin-5	B cell growth and eosinophil activation	Lopez 1988
IL-6	Interleukin-6	Metabolic - ↑ hepatic glucose output; ↑ GLUT4 transporters	Banzet 2009
		↑ hypothalamo-pituitary-adrenal axis to increase cortisol	Eskay 1990
		Leukocyte mobilization	Paulsen 2005; Yamada 2002
		↑ secretion of IL-1ra and IL-10	Steensberg 2003
		IGF suppression; myofibrillar protein loss	Caiozzo 1996
		Satellite cell proliferation	Hawke 2001
IL-8	Interleukin-8 (CXCL8)	↓ neutrophil infiltration via inhibition of adhesion to endothelial	Gimbrone 1989
		Upregulates monocyte infiltration	Li 2001
		Angiogenesis	Frydelund-Larsen 2006
IL-10	Interleukin-10	Anti-inflammatory, macrophage phenotype switch	Allavena 1997
		↓ Th2 response	De Waal 1991; Fiorentino 1991
		↓pro-inflammatory cytokine production	Murray 2006; Schroder 2003
IL-13	Interleukin-13	hypertrophy; MHC formation	Prokopchuck 2007
		↑ Expression of IL-8 by neutrophils; modulate neutrophil activity	Prokopchuck 2007
IL-15	Interleukin-15	↑ myosin heavy chain synthesis in presence of IGF-1	Cannon 1998
IL-17	Interleukin-17	↑ pro-inflammatory cytokine production; T cell differentiation	Onishi 2010

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MCP-1	Macrophage chemoattractant protein-1	Activation and recruitment of macrophages Restore muscle function following injury	Shireman 2007; Lu 1998 Warren 2004
M-CSF	Macrophage colony stimulating factor	↑ monocyte production in bone marrow	Zsebo 1988
MIP-1α	Macrophage inflammatory protein-1alpha (CCL3)	monocyte recruitment into injured tissue	Low 2001
MIP-1β	Macrophage inflammatory protein-1beta (CCL4)	monocyte recruitment into injured tissue	
TNFα	Tumour necrosis factor alpha	Activate proteolysis in presence of cortisol ↑ ICAM-1 and VCAM-1 expression ↑ IL-1β and IL-6 secretion Satellite cell proliferation	Mealy 1990 Cannon 1998 Galluci 1998 Li 2003

Footnote: This table includes possible actions of the cytokines previously observed to increase in serum of healthy young men following acute exercise *(refer to table 2.1)*. Abbr: ↑ - upregulate, ↓ - downregulate/ inhibit, ICAM – intracellular adhesion molecule, VCAM – vascular cell adhesion molecule.

2.2 Skeletal muscle regeneration

2.2.1 Exercise-induced muscle damage: a model to study regeneration

Characteristic signs observed following unaccustomed exercise are the immediate loss of force and muscle weakness followed by a more delayed onset of muscle soreness (DOMS). In 1939 Katz *et al.* noticed that when skeletal muscle is stretched beyond its optimum length it becomes weaker, explaining the loss of muscle force, whereas DOMS is directly associated with the forced lengthening of muscles while they are contracting *(eccentric contractions)* (Ammussen *et al*. 1952,1956). The magnitude of damage has been proposed to be related to the number of eccentric contractions (McCully *et al.* 1986, Warren *et al.* 1993), length of muscle stretch (Lieber *et al.* 1993, Brooks *et al.* 1995), tension generated (McCully *et al.* 1986) and the velocity of shortening (Chapman *et al.* 2006). It is thus not surprising that different exercise protocols vary substantially. In severe cases the destruction phase of skeletal muscle damage (*for reviews see* Jarvinen *et al.* 2005, Proske *et al.* 2001) is characterized by the rupture and necrosis of the myofibres and damage to the capillary bed leading to development of haematoma between the injured fibres and below the muscle sheath.

Clear evidence of the morphological changes in humans following eccentric exercise was first observed by Friden *et al.* (1981). The authors examined biopsies from subjects experiencing DOMS after running down many flights of stairs. Even though no signs of damage were evident using light microscopy, electron microscopy revealed disruption of the sarcomere structure specifically broadening and streaming of the z-lines (Friden *et al.* 1981).

Despite a lack of experimental evidence, researchers hypothesized that the non-uniform lengthening of sarcomeres (Huxley *et al.* 1961, Julian *et al.* 1979) might explain the mechanism behind eccentric exercise induced-muscle damage. In 1990 Morgan *et al.* proposed the "popping sarcomere hypothesis". The authors used an A.V Hill type model that allows for some random variation in the properties of sarcomeres to study the lengthening of active muscle and established that the more a sarcomere is stretched out the less tension can be generated since fewer cross-bridges form at longer sarcomere length. They further demonstrated that in their model rapid uncontrolled elongation to such an extent that there is no overlap between the thick and thin filaments *("popped sarcomere")* would occur in the weakest sarcomeres first. As result these weak sarcomeres would become even weaker, while the stronger sarcomeres remain able to return to their normal length. This theory led to the prediction that *in vivo* non-uniform lengthening of muscle fibres would occur during eccentric contractions and as a result of "popped weak sarcomeres" more tension would be placed on the stronger sarcomeres during repeated eccentric contractions. A crucial point the authors made was that even though the shortest sarcomeres are concentrated at the ends of a muscle fibre, these are not necessarily the weakest sarcomeres. They further hypothesized that the weak sarcomeres are scattered throughout the length of the muscle and that would explain non-uniform lengthening during eccentric exercise. This hypothesis was, however, only proven 6 years later. In 1996, Talbot & Morgan *et al.* counted the number of overstretched sarcomeres in a toad muscle fixed during an eccentric contraction and demonstrated the presence of overstretched sarcomeres scattered throughout the muscle.

A complicating factor in extrapolating this evidence to humans is that human skeletal muscle consists of three fibre types that may not be equally activated or equally prone to damage during eccentric exercise (Takekura *et al.* 2001, Macaluso *et al.* 2012). The proportion in which different fibre types are present within a specific muscle determines the overall contractile property of that muscle (Saltin *et al.* 1977). The three types of skeletal muscle fibres that can be identified in humans include: Slow-twitch oxidative fibres (Type I) which have a low myosin ATPase activity and high oxidative capacity (Dubowitz *et al.* 1960,

Brooke *et al.* 1970, Ahmadi *et al*. 2008), fast-twitch oxidative fibres (Type IIa) with moderate myosin ATPase activity and high oxidative capacity, fast-twitch glycolytic fibres (Type IIx) with high myosin ATPase activity and high glycolytic capacity (Brooke *et al.* 1970). Animal studies have demonstrated that type II fibres are more susceptible to eccentric exercise induced injury than type I fibres (Takekura *et al.* 2001). The reason for this difference in susceptibility to eccentric exercise induced damage between fibre types is still largely unknown, but it is thought to involve both mechanical and metabolic characteristics.

Rate of force development depend on the myosin ATPase reaction and the velocity of shortening. Type II fibres have a higher rate of force development, sarcomeres are thus unlikely to be synchronised during contraction. Fiber types also differ in terms of some structural proteins such as the wider Z-bands in human slow fibres, the narrow Z-bands in fast fibres may be structurally weaker (*for review see* Bottinelli *et al.* 2000).

Morgan *et al.* (1999) furthermore clearly state in a review on stretch-induced muscle damage, that the damage will depend on the sarcomere length range over which each fibre is stretched and that it varies with fibre type in mixed muscles. Individual variations in fibre type composition of the same muscle exist due to factors such as genetic inheritance, exercise, environment, detraining and disuse (Botinelli *et al.* 2000). Determination of individual subject's fibre type should be considered an important component in studies of eccentric muscle damage and this should be considered a possible confounding factor for interpretation of results of previous studies especially where results were unclear.

Primary skeletal muscle damage is not only due to mechanical disruption of structural proteins but also the sarcolemma. The loss of sarcolemmal integrity causes proteins *(such as creatine kinase (CK), myoglobin (Mb), fatty acid binding protein (FABP), lactate dehydrogenase (LDH) and fragments of myosin heavy chain (MHC) (Friden et al. 1989, Mair et al. 1992,Melin et al. 1997, Newham et al. 1983, Sayers et al. 2003))* to leak out and appear in circulation. However, it isn't clear to what extent the appearance of these proteins in circulation represent the extent of the initial insult, or whether they include an indication of secondary damage due to later destruction in response to the primary damage. Although frequently acknowledged, very few studies have been able to interpret the variability in elevation of these markers or their fluctuation over time since measurements are rarely taken at regular intervals especially from day 3 onwards.

Overstretching of sarcomeres may lead to damage to the t-tubules and/or sarcoplasmic reticulum causing alterations in excitation-contraction coupling (E-C coupling) leading to disruption of intracellular calcium (Ca^{2+}) homeostasis (Chen *et al*. 2007). The sustained Ca^{2+} influx into injured fibres can activate various proteolytic pathways with the ability to cause secondary muscle damage. Alderton & Steinhardt (2000) concluded, based on observations from studies using dystrophic muscle cells with abnormally active Ca^{2+} leak channels, that Ca^{2+} dependent proteolysis *(activity of calpains)* can cause degeneration by cleaving myofibrillar and cytoskeletal proteins. In addition, if Ca^{2+} homeostasis is not restored within a few days following severe EIMD, the damage to myofibers and cytoskeleton may become irreparable and it could lead to segments of the myofibre becoming necrotic (Allen *et al.* 2001, Friden *et al.* 2001, Gissel *et al.* 2001, Raastad *et al.* 2010). There is some controversy surrounding this as a mechanism for secondary muscle damage following exercise. Calcium homeostasis could be disrupted in fatigued muscles without the presence of any structural damage. Association between direct evidence of damage *(such as z-line streaming)* and indirect markers of damage *(such as DOMS and the presence of muscle proteins in circulation)* is substantial, but not quantitative and mechanisms are poorly understood. This could be due to the number of different protocols used to induce muscle damage.

Many studies of eccentric exercise induced skeletal muscle damage and the subsequent inflammatory response, carefully eliminate all concentric components from the exercise protocol. Skeletal muscle damage induced by purely eccentric contractions is thus an isolated event specific to the exercising muscle and usually occurs without the presence of metabolic or cardiovascular fatigue. Downhill running on the other hand is a much more functional activity combining concentric and eccentric exercise resulting in the release of pro-inflammatory cytokines associated with both damage associated molecular patterns (DAMPs) and metabolic stress.

When running against a negative slope the contracting quadriceps muscle group controls the rate of knee flexion against the force of gravity. The externally imposed tension requirement on the muscle exceeds the tension that would develop at a constant muscle length (Raven *et al.* 1991, Morgan *et al.* 1999) and the muscle lengthens while it is contracting. In the laboratory setting the downhill protocol can be manipulated to achieve the desired effect. The greater the negative slope the greater external tension imposed on the muscle while the speed of the treadmill causes additional stress imposed by the rate of strain development. The

duration of the downhill running adds a third dimension, metabolic fatigue, whereas intermittent downhill running may limit damage associated with metabolic stress.

Beaton *et al.* (2002) demonstrated that structural damage *(Z-line streaming)* and the presence of immune cells in biopsies from humans' *vastus lateralis* muscles following eccentric contractions is highly variable with a coefficient of variance (CV) of 40 to 70 %. The authors studied z-band disruption and the presence of immune cells in the *vastus lateralis* muscle of 5 healthy young men following maximal isokinetic eccentric contractions (*240 repetitions at 30 degree angle).* Two muscle biopsies were taken within the same muscle 24h post exercise and on a second occasion a single biopsy was taken from the contralateral leg, also 24h following the same damage protocol. The study demonstrated that the within-leg CV for Z-band disruption was 41 ± 30% and between legs 68 ± 36 % and immune cell infiltration between biopsies varied with 48 ± 27%. Large individual variability in response to eccentric exercise has been reported on numerous occasions (Chapman *et al.* 2008, Chen *et al*. 2006, Clarkson *et al*. 1992, Gulbin *et al*. 2002, Hubal *et al*. 2007, Newham *et al.* 1983, Nosaka *et al*. 1996, Paulsen *et al.* 2010, Sayers *et al.* 2001,2003, Sewright *et al.* 2008, van de Vyver *et al.* 2012). Therefore, subjects were classified into "low", "medium" and "high" responders based on changes in muscle function, CK activity and signs of necrosis. In several of the studies, these divergent response patterns to EIMD may represent an ideal model for identifying mechanisms of inflammation and a second phase of damage potentially induced by limited resolution of inflammation. Inflammation is, on the other hand involved in activation of regeneration. Particularly satellite cells are known to be influenced by pro-inflammatory cytokines.

2.2.2 Satellite cell response to exercise-induced muscle damage

Adult skeletal muscle consists of terminally differentiated tissue, but has remarkable regenerative capabilities. Since Mauro *et al.* (1961) discovered satellite cells (SC) *(muscle progenitor cells located between the sarcolemma and basal lamina of muscle fibres (Moss& Leblond 1970))* it has been known that their activation is crucial for skeletal muscle growth and/or repair. Satellite cells are induced to fuse with muscle fibres adding myonuclei in order to maintain the nuclear domain during growth/ hypertrophy; or to repair damaged fibres following injury. Generation of daughter cells replenishes the SC pool (*reviewed in* Charge *et*

al. 2004, Chen *et al.* 2003, Grounds *et al*. 2002, Hawke *et al*. 2001, Le Grand *et al.* 2007). With severe damage SCs may also fuse with each to form new myofibres (Hill *et al.* 2003).

Other stem cells *(including pericytes, mesoangioblasts, side population cells, haematopoietic progenitor cells and endothelial progenitor cells)* also have the capability of contributing to muscle regeneration (*reviewed in* Macaluso *et al.* 2012). The ability of non-muscle derived stem cells to engraft into the SC niche (Blaveri *et al.* 1999, Fukada *et al.* 2002) has been confirmed in animal models where bone marrow derived stem cells contributed to skeletal muscle regeneration following severe injury (LaBarge *et al.* 2002, Palermo *et al*. 2005). This was demonstrated when transplanted GFP-labelled bone marrow derived stem cells entered the satellite cell niche and contributed to skeletal muscle regeneration following irradiation and subsequent muscle damaging exercise. The same authors (Palermo *et al.* 2005) later used parabiotically joined wild type mice with genetically labelled bone marrow and indicated that stem cells originating in the bone marrow were evident in myofibres after one month of forced exercise *(extensor digitorum longus (EDL) overload)*. Lethal irradiation and bone marrow transplantation have formed a key role in the animal experimental models that therefore do not truly represent exercise-induced regeneration of skeletal muscle. The contribution of these non-muscle stem cells to skeletal muscle repair will therefore not be discussed further. After a short explanation of SC behaviour that leads to the ability to identify satellite cells in tissue, the literature review will proceed to the discussion of damage in humans.

When compared to myonuclei, quiescent SCs *(located in the satellite cell niche)* have a higher nuclear: cytoplasmic ratio with less organelles and a larger amount of heterochromatin (*reviewed in* Charge *et al.* 2004). The quiescent SCs are attached to the basal lamina *(integrin α7β1 interact with laminin)* and the adjacent myofibre *(interaction with m-cadherin)* (Boldrin *et al.* 2010, Gnocchi *et al.* 2009, Volonte *et al.* 2005). During SC transition from quiescent into activated cells, their nuclei enlarge and become euchromatic, the cytoplasm expands and the organelles *(rough endoplasmic reticulum and mitochondria)* start to enlarge (Schultz *et al.* 1985). The gene expression of these cells differs from that of myonuclei. Therefore proteins such as Ki67 and PCNA *(proliferating cell nuclear antigen)* are expressed.

Activated SC's re-enter the cell cycle and start to proliferate. Proliferation occurs in many cell types, but proteins expressed by cells with regenerative potential are also expressed in at

least a portion of satellite cells *(e.g DLK-1 and FA-1)* (*refer to table 2.5, p. 49*). Depending on factors influencing the niche, proliferating satellite cells proceed to myoblasts and further differentiate and may fuse. The progression of SC's through these phases is coordinated by the up- and downregulation of specific myogenic regulatory factors (MRF's).

The involvement of hepatocyte growth factor (HGF) in SC activation has previously been shown (Allen *et al.* 1995) and is thought to initiate the sphingolipid signalling cascade (*reviewed in* Macaluso *et al.* 2012). HGF binds to the extracellular matrix of SC's or interacts directly with the c-met receptor located on the SC surface membrane (Cornelison *et al.* 1997). Tatsumi *et al.* 2002 therefore suggested that the co-localization of HGF and c-met receptors can be used as an early indicator of SC activation.

Based on early animal model experiments it is known that the mitotic activity of satellite cells occurs mainly near the site of injury, but SC along the whole fibre can migrate to the injured area. Schultz *et al.* (1985) monitored SC mitotic activity through autoradiography after ^{3}H-thymidine injection following a crush injury to rodent *extensor digitorum* muscles. The authors established a gradient along the muscle length and demonstrated that activation of SCs is not restricted to the damaged area. These active SCs migrate to the site of injury under the basal lamina or along adjacent fibres depending on the integrity of the basement membrane (Schultz *et al.* 1986).

Table 2.5 Satellite cell markers

		Satellite cell expression				
Antigen	**Expression**	**Quiescent**	**Proliferating**	**Differentiating**	**Function**	**First Auth, date**
CD34	Stem cells; surface	+	±	-	Modulation of cell activity	Alfaro 2011; Beauchamp 2000
c-met	SC surface; sarcolemma; interstital cells	+	+	-	Receptor for HGF	Lindstrom 2010; Cornelison 1997
DLK-1 *(Pref-1/pG2)*	portion of SC; interstitial cells	+	+	-	precurser for FA-1	Lee 1995; Lindstrom 2010;
FA-1	All undifferentiated cells	±	+	-	Marker for cells with regenerative potential	Floridon 2000
Ki-67	All proliferating cells	-	+	-	Indicative of proliferation	Bridger 1998; Mackey 2009
m-cadherin	Ca2+ dependant homophilic cell adhesion molecule	±	+	+	Alignment and fusion of myoblasts	Cornelison 1997; Donalies 1999
MRF4	SC nuclei; myoblasts	-	-	+	MRF - differentiation and fusion	Cornelison 1997
Myf-5	SC nuclei	±	+	-	MRF - migration and SC self-renewal	Beauchamp 2000; Cornelison 1997
MyoD	SC nuclei;myoblast nuclei	-	±	+	MRF - trigger for terminal differentiation	Cornelison 1997
Myogenin	SC nuclei;myoblast nuclei	-	-	+	MRF- differentiation and fusion	Lindstrom 2010
NCAM *(CD56/Leu-19)*	surface embryonic myotubes; SC surface	+	+	-	developmental molecule	Fidzianska 1995
Pax7	SC nulei	+	+	-	Indicative of proliferation	Zammit 2004
PCNA	All proliferating cells	-	+	-	Indicative of proliferation	McKay 2009

Footnote: Table include markers used to identify satellite cell activity in humans following acute exercise.
Abbr: CD- cluster of differentiation; DLK- delta-like homoloque; FA- fetal antigen; HGF- hepatocyte growth factor; MRF- myogenic regulatory factor; NCAM - neural cell adhesion molecule; PCNA- proliferating cell nuclear antigen, Pref- preadipocyte factor, SC - satellite cell.

MRF's bind to a consensus DNA sequence *(called an E-box)* and form heterodimers with ubiquitously expressed E-protein family members *(including E12/47, E2-2, E2-5)*, and regulate the expression of several muscle-specific and cell cycle regulatory genes (Kataoka *et al.* 2003).

In vitro single-cell analysis of regulatory gene expression indicated that an increase in the level of MyoD is evident just before the onset of differentiation (Cornelison *et al.* 1997). MyoD induces the expression of cyclin-dependent kinase inhibitor *(p21WAF1/Cip1)* resulting in exit from the cell cycle. Exit from cell cycle is the first prerequisite for differentiation (Halevy *et al.* 1995, Martenelli *et al.* 1994). However, some SC return to quiescence. The MRF, myf-5 is thought to play a role in promoting SC self-renewal (Cornelison *et al.* 1997). Particularly when its expression dominates that of myoD. In contrast, when myoD expression dominates, it is associated with the upregulation of myogenin *in vitro* (Olguin *et al.* 2007). It is therefore concluded that myoD promotes the progression of proliferating SC's to start terminal differentiation. Differentiating SC's also express the myogenic regulatory factor-4 (MRF-4), thought to play an important role in fusion and the formation of new myofibres (Cronelison *et al.* 1997, Le Grand *et al.* 2007).

Depite *in vivo* experiments *(mainly animal models using electron microscopy and/or nucleus labelling with BrdU)* clearly demonstrating the incorporation of SC's into damaged fibres, our understanding of the mechanism and specific factors involved in SC activity in humans are mainly based on evidence from *in vitro* models. Also, the satellite cell response to a single bout of unaccustomed exercise is highly variable (*refer to table 2.6, p. 51*) and does not seem to progress similarly to bigger injuries induced in small animal models.

Table 2.6 Satellite cell response to acute muscle damaging exercise.

First Auth, date	Intervention protocol	Subjects	biopsy time points	Detection	Satellite cell response
Malm 2000	eccentric cycling	healthy young men	baseline, post, 6h, d1,d2,d4,d7	NCAM	2.7 %↑SC/fibre (48h) in ex leg; 2% ↑SC/fibre (24h) control leg
Crameri 2004	eccentric contractions quadriceps (8 reps of 10 one-leg drop down jumps)	healthy young men	5h, d2,d4,d8	NCAM	± 192%↑SC/myonuclei (d4)
				FA1	± 192% ↑ FA-1^{+} mononuclear cells (d4)
				myogenin	No change
Dreyer 2006	maximal knee extensor ex (6 sets of 16 reps)	young men vs old men	baseline, d1		± 141%↑ SC / myonuclei (d1)
Crameri 2007	210 maximal ecc contractions quadriceps	untrained men	baseline, 5h,d1,d4,d8	NCAM	5% ↑SC/myonuclei (d8)
				Pax7	2% ↑ SC/myonuclei (d4)
				myogenin	no change
O'Reilly 2008	300 maximal ecc contractions quadriceps	recreationally active males	baseline, 4h, d1, d3,d5	NCAM	80% ↑SC/myonuclei (d3)
McKay 2009	300 maximal ecc contractions quadriceps	healthy young men	baseline, 4h, d1, d3,d5	Pax7	6% ↑ SC/ myonuclei (d1)
Mikkelsen 2009	200 maximal ecc contractions	healthy young men	baseline, d8	Pax7	96% ↑ SC/ fibre (d8)
McKay 2010	300 maximal ecc contractions	healthy young men	baseline,d1	Pax7/ NCAM	36% ↑ flow cytometry analysis (d1)
				Pax7/ NCAM	28-36% ↑IHC analysis (d1)
Paulsen 2010	70 maximal ecc contractions elbow flexors	healthy young men and women	baseline, 1h,d2,d4,d7	NCAM/ Ki-67	combined (1h-d7) ↑in ex group than control group
Snijders 2012	single bout combined endurance resistance ex	healthy young men	baseline, post, 9h	DLK1	19% ↑ in type I fibres (9h); 27%↑ in type II fibres (9h)
				Ki-67	no change
van de Vyver 2012	10% DHR (12 x 5min bouts) @ 75% VO2max	recreationally active males	baseline, d1,d2	Pax7	30% ↑SC/ fibre (d1)

Footnote: Table include all studies that measured changes in satellite cell activity following a single bout of muscle-damaging exercise in healthy young men. **Abbr**: ↑- increase,↓- decrease, d- day, ex- exercise, h- hour, SC- satellite cell.

In addition to the effect of age (Dreyer *et al.* 2006), fibre type, cross-sectional area, history of muscle injuries and even regular physical activity might influence the number of SCs present within a specific muscle. Based on evidence from animal models showing that type II fibres are more susceptible to damage from eccentric contractions, it is possible that more SCs will be associated with type II than type I fibres. Human *vastus lateralis* muscle is most commonly biopsied. The number of quiescent SCs seem to be equally distributed between fibre types (Kadi *et al.* 2006). Macaluso *et al.* (2012) investigated the substantial variability in SC content among young individuals and hypothesized that an active lifestyle may be associated with greater baseline SC numbers. The authors demonstrated that SC number *($Pax7^+$)* is proportional to VO_2max, suggesting that higher levels of physical activity *(even without injury)* activates SC proliferation but not fusion. This study indicated no correlation with fiber type or myogenin. Snijders *et al.* (2012) also demonstrated no difference in the percentage of SCs *($DLK1^+$ and $Ki\text{-}67^+$)* between fibre types at baseline. The authors then demonstrated that despite increases in $DLK1^+$ cells *(19% increase in type I and 27% increase in type II fibres)*, following a single bout of combined endurance and resistance exercise, no significant changes were observed in SC content. This could imply that existing SCs were activated and began to express DLK1.

This is supported by findings from Mackey *et al*. (2007), demonstrating that SCs are triggered to become active in response to resistance training without the presence of damage. Taken together these studies suggest that SCs activated to proliferate in response to acute exercise will simply return to the quiescent state unless stimulated to a greater extent or over a longer period of time. In (2004) Zammit *et al.* proposed a model for renewal of the satellite cell pool involving the expression of the paired box transcription factor, Pax7. The authors used live cell imaging through fluorescent microscopy to observe the activity of isolated mouse primary myoblasts with different expression profiles *($Pax7^+/MyoD^-$, $Pax7^+/MyoD^+$, $Pax7^-/MyoD^+$)*. A small population of SCs that maintained the expression of Pax7 *($Pax7^+/MyoD^-$)* returned to quiescence. In agreement, Olguin *et al.* (2007) later demonstrated that the overexpression of Pax7 downregulates MyoD and therefore represses myogenesis.

Various factors associated with muscle contraction or in the case of EIMD, factors associated with the subsequent inflammatory response are known to affect SC activity *in vitro*. The following paragraphs will discuss the evidence implicating an interaction between inflammatory mediators and SC activity in humans.

In human EIMD models, increases in serum pro-inflammatory cytokines *(refer to table 2.1, p. 27)* coincide with SC proliferation *(refer to table 2.6, p. 51*) during the early recovery phase. van de Vyver *et al.* (2012) demonstrated that a 30% increase in $Pax7^+$ SCs one day following intermittent downhill running *(12 x 5min bouts @ 75% VO_2max)*, coinciding with increases in serum IL-6 and TNFα concentrations. Based on evidence implicating IL-6 in SC-mediated hypertrophy in animals (Serrano *et al.* 2008), McKay *et al.* (2009) investigated the role of IL-6 in the SC response to eccentric exercise in humans. The authors demonstrated an increased expression of STAT3 responsive genes *(cyclin D1 and SOCS3)* and the expression of IL-6 within SCs themselves that correlated with an increase in SC proliferation ($Pax7^+/PCNA^+$) following muscle lengthening contractions. Toth *et al.* (2011) confirmed this observation using immunohistochemistry to demonstrate that IL-6 induced STAT3 signalling occurs exclusively in the nuclei of SCs in healthy young men in response to muscle lengthening contractions *(300 maximal eccentric contractions, quadriceps)*.

There is however a lack of evidence showing SC differentiation and fusion in the human exercise model. Various studies have failed to detect changes in intramuscular myoD and/or myogenin protein levels despite increased SC proliferation (Crameri *et al.* 2004, 2007, Dreyer *et al.* 2006, Kadi *et al.* 2004) in response to acute exercise. This supports the notion by Macaluso *et al.* (2012) that physical activity stimulates the SC pool size. However, in the study of van de Vyver *et al.* (2012) SC proliferation was not sustained 2 days after one acute bout of downhill running, indicating that regular activity would be required to maintain a greater pool size. It is thus possible that even though pro-inflammatory mediators promote SC proliferation, sustained inflammation *(associated with the release of cytokines and growth factors by immune cells)* might be delaying or even inhibiting myogenesis.

NFκB is often referred to as the "central mediator of the immune response". Based on *in vitro* and animal model experiments it furthermore seems that SC proliferation is promoted and differentiation inhibited by classical intramuscular NFκB signalling (*refer to section 2.3.3, p. 58*). The next section of this review will therefore focus on a basic understanding of the NFκB signalling pathway and on the involvement of this signalling pathway in the *in vivo* context of exercise.

2.3 Nuclear Factor Kappa-B (NFκB) signalling pathway

Since muscle damaging exercise is associated with severe cellular stress and an acute inflammatory response, it is not surprising that exercise physiologists began to show interest in NFκB signalling. To date the majority of muscle related NFκB signalling studies have been done in cell culture using either mouse embryonic fibroblasts (MEF), murine C2C12 or rat L6 cell lines. These studies greatly contribute to our understanding of the various NFκB signalling pathways.

The NFκB signalling pathway is an intricate network of inhibitor proteins and transcription factors. In 1999 Pahl *et al.* reviewed the literature published between 1989 and 1999 and compiled a list consisting of over 150 known activating signals and NFκB target genes. Based on this comprehensive list, it became apparent that the NFκB transcription factor seems to be involved in various aspects of the immune system with upstream activating signals including inflammatory cytokines, oxidative stress, mitogens and bacterial products (Pahl *et al.* 1999, Bakkar *et al.* 2010). The authors further concluded that the vast majority of NFκB inducing agents represent a form of stress to cells and that in response many NFκB target genes function to alleviate cellular stress.

2.3.1 NFκB and IκB family members

There are five genes that encode the NFκB family of proteins. These include RelB, RelA (p65), cRel, p50 also known as NFκB1 *(derived from precursor p105)* and p52 also known as NFκB2 *(derived from precursor p100)*(Ghosh *et al.* 1998, Mourkioti *et al.* 2008, Bakkar *et al.* 2010).

Since the first discovery of NFκB, the structure and function of its family members *(also referred to as subunits)* have been extensively investigated, and reviewed in the literature on many occasions (Grilli *et al*. 1993, Kopp *et al*. 1995, Verma *et al.* 1995, Ghosh *et al.* 1998, Heyden *et al.* 2004, Mourkioti *et al.* 2008, Bakkar *et al.* 2010). The first NFκB molecule described was a heterodimer of p50 and p65 subunits (Kopp *et al*. 1995, Verma *et al.* 1995). This protein is still what is commonly referred to as NFκB despite the diversity of other hetero- and homodimers that are now known to also form part of the NFκB family.

Each one of the NFκB subunits contain a 300 amino acid N-terminal domain, the Rel homology domain (RHD). The RHD is responsible for interaction between subunits and subsequent formation of hetero- and/or homodimers as well as mediating binding with promoter regions containing –κB sites (*reviewed in* Bakkar *et al.* 2010). Upon further investigation it became clear that NFκB dimers seem to bind with different affinities to target genes. In 1991 Schmitz *et al.* indicated that the p65 subunit and therefore heterodimers such as p65/p50 and p65/p52 induce gene expression and are therefore transcriptionally active. Many years later, Tong *et al.* (2004) indicated that homodimers such as p50/p50 and p52/p52 do not have transactivation domains are in fact transcriptionally repressive. It was further demonstrated that the heterodimers can be phosphorylated at different amino acids *(serines)* which will determine their interaction with transcriptional modulators as well as any post translational modification that may occur (Duran *et al.* 2003, Campbell *et al.* 2004, O'Shea *et al.* 2008). The upstream activating signal will therefore determine which serine is phosphorylated and thus ultimately how gene transcription will be affected by a specific NFκB dimer.

In the absence of an activation stimulus, the NFκB heterodimers are kept inactive in the cytosol via association with an NFκB inhibitor protein, known as IkappaB (IκB). When this inhibition is lifted, IκB is phosphorylated and targeted for degradation. (Ghosh *et al.* 1998, Mourkioti *et al.* 2008, Bakkar *et al.* 2010). In the inactive state, the IκBα/p65/p50 complex is however still able to shuttle between the cytoplasm and nucleus (Ghosh *et al.* 2002, Bakkar *et al.* 2010) to maintain a basal level of NFκB induced transcription.

NFκB signalling can be mediated by two very distinct pathways: the classical *(canonical)* activation pathway and the alternative *(non-canonical)* activation pathway. In the classical pathway the NFκB heterodimer (p65/p50) translocates to the nucleus (Beg *et al.* 1993) and induces gene transcription while the alternative pathway ends with RelB/p52 complex assembly and translocation to the nucleus.

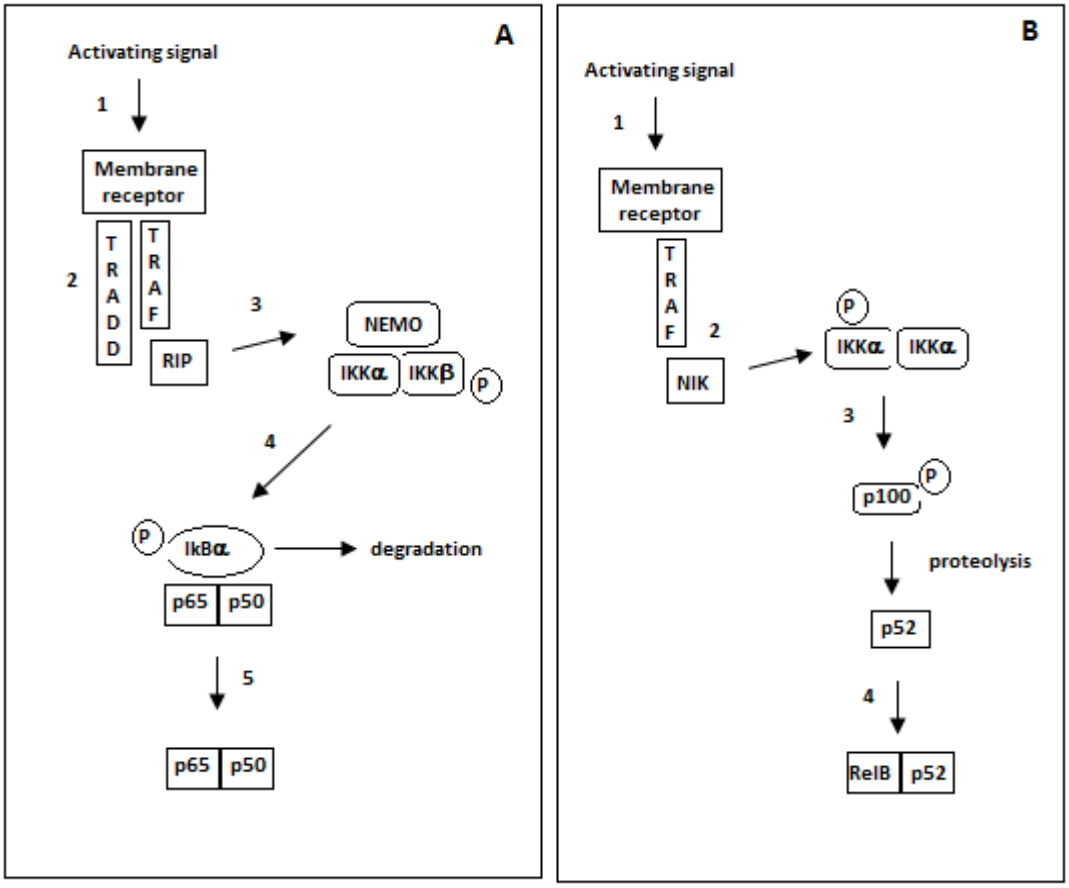

Fig 2.1 NFkB signalling. **A: classical activation of NFκB**.**1**. Activating signal binds to its membrane receptor **2**. Adaptor proteins (TRADD), receptor associated factors (TRAF) and receptor interacting proteins (RIP) are recruited to the membrane **3**. RIP binds to NEMO and result in IKKβ phosphorylation **4**. IκBα is phosphorylated by IKK complex and targeted for degradation 5. p65/p50 heterodimer translocate into the nucleus. **B: alternative activation of NFκB** 1. Activating signal binds to its membrane receptor and recruit receptor associated factors (TRAF) 2. Activated NIK phoshorylates IKKα homodimer 3. p100 is phosphorylated and p52 generated through partial proteolysis 4. RelB/p52 complex translocates to the nucleus.

2.3.2 Effect of acute exercise on circulating leukocyte NFκB signalling

It is clear from the previous sections of this literature review that muscle and the immune system are very responsive to one another. Skeletal muscle is not the only tissue in which NFκB signalling is activated during exercise. In 2001 Vider *et al.* were the first to demonstrate the effect of physical activity on *in vivo* human leukocyte NFκB signalling . The

authors demonstrated that an increase in the plasma concentrations of NFκB gene products *(TNFα and IL-2 receptor)* coincided with the presence of transcriptionally active p65 subunits inside peripheral blood lymphocytes. Suggesting that acute exercise *(1 hour physical exercise @ 80% VO_2max)* triggered the classical activation of NFκB in healthy young men.

Acute muscle damaging exercise has also been shown to activate leukocyte NFkB signalling. Garcia-Lopez *et al.* (2007) indicated increased NFκB activation coinciding with increased p-IκBα and p-IKK protein levels inside peripheral blood mononuclear cells (PBMC's) following an acute bout of eccentric exercise *(12 sets of 10 barbell sqauting)*. More importantly the authors were able to demonstrate that these changes in NFκB signalling were significantly attenuated by six weeks of eccentric training. Similarly, Jimenez-Jimenez *et al.* (2008) demonstrated that a significant increase in p65/p50 activity and the phosphorylation of IκB in PBMC's following unaccustomed exercise were reduced with 8 weeks of eccentric training in the elderly. In addition the authors indicated a similar reduction in the mRNA expression of iNOS, IL-6 and cyclooxygenase-2 (COX-2) after training, indicating that the undesired inflammatory response was prevented. Those data provide evidence to suggest that PBMC NFκB signalling may be responsible, at least in part, for triggering the acute inflammatory response following muscle damaging exercise.

In contrast to the study of Garcia-Lopez *et al.* (2007), Rietjens *et al.* (2007) demonstrated that despite increases in overall antioxidant capacity, the activity of NFκB in PBMC's remained unchanged and no inflammatory response was evident following a single session of resistance exercise *(8 sets of 10 leg press and extensions)*. A possible explanation for this contradiction might be the association between NFκB signalling and exercise intensity as observed by Kim *et al.* (2009). The authors demonstrated a direct link between the intensity *(% of reserved heart rate)* of concentric treadmill exercise and NFκB nuclear binding activity in PBMCs. Maes *et al.* (2007) have furthermore demonstrated that PBMC NFκB signalling is responsible for the inflammatory response associated with chronic fatigue syndrome .

The involvement of NFκB signalling in the induction of pro-inflammatory gene expression in PBMCs after exercise is thus highly likely. Specific evidence of a direct link in human exercise models is still lacking.

2.3.3 Intramuscular NFκB signalling

Experiments focussing on NFkB signalling in muscle have taken two quite different approaches. Cell culture *(mainly C2C12, myoblasts, MEF)* experiments focussed on effects on proliferation and differentiation whereas *in vivo (animal and human models)* considered inflammation. Few studies in cells, animals or humans focussed on damage and regeneration.

Baeza-Raja *et al.* (2004) demonstrated a p38 mitogen activated protein kinase (MAPK) *(promyogenic factor)* dependant elevation of p65/p50 DNA binding activity and that this specific NFκB activation is required for IL-6 production. The authors further demonstrated that transfection of C2C12 cells with specific IL-6 silencing RNA's (siRNA's) result in decreased myogenin mRNA levels indicating that IL-6 expression is essential for efficient muscle differentiation. In agreement, decreased IκB ubiquitination, NFκB nuclear translocation and inhibition of differentiation were associated with one another following 3D clinorotation to simulate microgravity in L6 myoblasts (Hirasaka *et al*. 2005).

On the other hand, several activators of NFκB such as TNFα, TWEAK, RIP2 and IL-1β is known to strongly inhibit myogenesis (Dogra *et al.* 2006, Girgenrath *et al.* 2006, Guttridge *et al.* 2000, Langen *et al.* 2001, Munz *et al.* 2002). By stimulating C2C12 cells with different concentrations *(0.1-10ng/mL)* of TNFα and IL-1β Langen *et al.* (2001) demonstrated an increase in NFκB activity that ultimately inhibited differentiation. In support of this finding several other studies have also shown that sustained NFκB activation impair differentiation, and that there is an overall decline in NFκB DNA binding during differentiation (Bakkar *et al*. 2005, Dee *et al*. 2003,Guttridge *et al.* 1999, Lehtinen *et al.* 1996) under normal conditions.

The precise mechanisms by which classical NFκB signalling inhibit myogenesis is still unclear but several possibilities have been proposed. TNFα induced NFκB activation *in vitro* have been shown to decrease MyoD synthesis and simultaneously destabilize this myogenic regulatory factor (Dogra *et al.* 2006, Guttridge *et al.* 2000), thereby limiting differentiation. Wang *et al.* 2007 furthermore indicated that classical NFκB signalling activates YY1 *(transcriptional repressor protein)* that in turn represses the transcription of muscle structural proteins such as troponin, myosin heavy chain and α-actin . It is important to note though that the TNFα concentrations used to induce classical NFkB signalling *in vitro* is above the physiological ranges and that TNFα induced intramuscular NFκB signalling has not yet been confirmed in human exercise models.

Animal experimental models mainly supports the findings of cell culture experiments showing that classical NFκB signalling has a negative effect on myogenesis. Mourkioti *et al.* (2006) demonstrated that IKK2 depleted mice show enhanced regeneration and faster clearance of inflammation following denervation when compared with wild type mice. This has been supported by various *in vivo* animal studies indicating that NFκB signalling contributes to skeletal muscle damage and/or wasting (Judge *et al.* 2007, Bakkar *et al.* 2008, Evans *et al.* 2010). In 2009, Chiang *et al.* demonstrated that downregulation of NFκB signalling via honokoil exhibits protective effects against exercise-induced skeletal muscle damage in rats following repeated days of downhill running. The authors suggested that this protective effect was due to the modulation of inflammation-mediated damage to muscle cells.

Based on the evidence from animal models, Durham *et al.*(2004) hypothesized that skeletal muscle contraction leads to NFκB activation that ultimately promotes protein catabolism. In contrast to their hypothesis, 45 minutes of resistance exercise *(single repetitions of progressively greater weights)* in humans resulted in a significant decrease in NFκB activity immediately post exercise that returned to baseline values following one hour of bed rest. This finding was supported by an *in vitro* model, where the authors isolated the *soleus* and diaphragm muscles from animals and showed a 44% decrease in NFκB activity following 10 min of *in vitro* electrical stimulation (Durham *et al.* 2004). In a third model using male ICR mice, the authors indicated that an increase in NFκB activity due to 12 days of hindlimb unloading can be reversed by 10 min of contraction *in vitro*. Based on these findings the authors proposed that acute fatiguing exercise reduces NFκB activity to protect the exercising muscles against wasting.

Contradicting these findings, Vella *et al.* (2011) demonstrated an increase in NFκB p65 protein levels and nuclear localization in the *vastus lateralis* muscle of recreationally active males 2 hours following a single bout of high intensity resistance exercise. Vella *et al.* (2011) furthermore illustrated an increase in NFκB binding to MCP-1, IL-6 and IL-8 promotor regions coinciding with increases in the mRNA expression of these cytokines. Thereby implicating NFκB in the transcriptional control of cytokines known to be central to the post–exercise inflammatory response.

On the other hand various studies have shown no change in intramuscular NFκB activity following a variety of exercise protocols. In 2007 Nieman *et al.* demonstrated that regardless

of supplementation with an antioxidant *(quercetin)*, 3 hours of moderate intensity cycling per day for 3 consecutive days did not increase muscle NFκB activity in elite cyclists. Focussing on intramuscular gene expression following downhill running, Buford *et al.* (2009) were also unable to detect any change in NFκB activity in the first 24 hours following exercise in sedentary males.

In summary, pro-inflammatory TNFα, IL-1 and NO activate NFκB activity in leukocytes and induce the expression of additional TNFα, IL-1 and IL-6. TNFα and IL-1 in turn also activate intramuscular NFκB signalling. TNFα and IL-1 also activate intramuscular p38 MAPK signalling known to increase myoD activity, the expression of myogenin and promote fusion. IL-6 on the other hand is known to promote myoblast proliferation through STAT3 signalling *(refer to the next section 2.4).*

2.4. Intramuscular Signal transducer and activator of transcription (STAT) signalling

There are seven STAT family members *(STAT 1, 2, 3, 4, 5A, 5B and 6)* that transduce the effects of various cytokines and hormones (Levy *et al.* 2002). The IL-6 family of cytokines *(IL-6, leukaemia inhibitory factor (LIF), ciliary neurotrophic factor, oncostatin M (OSM), IL-11, cardiotrophin-1)* and various growth factors *(insulin like growth factor-1 (IGF-1), HGF, epidermal growth factor, platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF))* activate STAT3 transcriptional activity and play a central role in the regulation of growth, differentiation and cell survival (Hirano *et al.* 2000).

The downstream effects of IL-6 is dependent on its interaction with either a membrane bound (gp130) or soluble receptor (sIL-6R) (Robson-Ansley *et al.* 2010). Binding of the IL-6 family of cytokines to their receptors leads to the homodimerization of gp130 or heterodimerization of gp130 with other gp130-related receptors *(LIF receptor β, OSM receptor α, CT-1 receptor α)* which results in the activation of the gp-130 associated Janus kinases *(JAK1, JAK2,Tyk2)*. Subsequently gp130 is phosphorylated on tyrosine and the phosphorylated molecule recruits signal-transducing molecules (STATs). STATs are normally located in the cytoplasm until it is activated through phosphorylation of its tyrosine residue (Rane *et al*. 2000). The tyrosine phosphorylation of STAT3 induces either STAT3 proteins to form homodimers or a STAT3 and another isoform of STAT to form a heterodimer with their phosphotyrosine-SH2 domain

interactions (Decker *et al.* 1999). The active STAT dimer then rapidly translocates to the nucleus by importin α/β localization pathway (Rane *et al*. 2000).

The functional role for STAT3 signalling in growth and differentiation is very diverse and seem to depend on the cell type and/or activating cytokine. In fibroblasts and hematopoietic cell lines, STAT3 promotes proliferation through the transcriptional regulation of cyclin D1 and c-myc respectively (*Bromberg et al. 1999, Kiuchi et al. 1999, Shirogane et al. 1999*). Following a comprehensive investigation into the role for STAT3 in the growth and differentiation of myoblasts, Kataoka *et al.* (2003) were able to demonstrate that STAT3 induce the expression of c-myc *(promoting proliferation) in vitro* and that it antagonizes MyoD through direct protein interaction. STAT3 has furthermore been shown to maintain embryonic stem cells in an undifferentiated state (Matsuda *et al.* 1999).

In agreement, IL-6 induction of STAT3 signalling have been shown to occur exclusively in the nuclei of intramuscular satellite cells (Toth *et al.* 2011) and correlates with increases in satellite cell proliferation in response to acute muscle lengthening contractions (McKay *et al.* 2009) in healthy young men. Taken together these results suggest that STAT3 signalling promote proliferation and simultaneously suppresses myogenic differentiation.

Contradicting these findings, Jang *et al.* (2012) recently indicated an elevation in STAT3 during differentiation that was further increased by JAK3 inhibition in the C2C12 myoblast cell line. The authors furthermore demonstrated that in addition to increased STAT3 signalling, JAK3 inhibition/ knock down *(WHIp54 inhibitor and siJAK3)* significantly increased the expression of myosin heavy chain (MHC), myogenin, myoD and myogenic enhancer factor 2 (MEF-2). Spangenburg *et al.* (2002) did however demonstrate that satellite cell proliferation *in vitro* induced by LIF *(member of IL-6 family of cytokines)* is mediated by the activation of the JAK2-STAT3 signalling pathway and does not involve JAK3.

On the other hand, IL-6 is known to be associated with muscle wasting in a variety of diseases and *in vivo* models (*reviewed in* van Hall *et al.* 2012). Bonetto *et al.* (2011) proposed a mechanism by which STAT3 causally influence muscle wasting associated with cancer. The authors used both cultured C2C12's and a C26 cachexia model in conjunction with micro array analysis to demonstrate an association between moderate and severe cachexia and an increase in the IL-6 induced STAT3 gene expression profile. STAT3 co-localization in myonuclei and an increase in phosphorylation status of STAT3 (Y705) were also evident *in vitro*.

It should be noted that most of the early information available on the downstream effects of IL-6 come from *in vitro* experiments where single recombinant cytokines and many different cell lines have been used. It is thus difficult to assess whether the observed *in vitro* effects reflect the *in vivo* situation. More recently, information regarding the *in vivo* action of IL-6 especially in the acute exercise and EIMD models have become available since the presence of IL-6 in circulation following acute exercise *(with or without skeletal muscle damage)* has been a consistent finding (*refer to table 2.1, page 27*).

There is however a lack of investigation into the intramuscular activity and function of STAT3 signalling in response to acute exercise-induced muscle damage. From table 2.7 *(p. 63)* it is clear that only McKay *et al.* (2009) and Toth *et al.* (2011) investigated the effect of IL-6 induced STAT3 signalling in response to EIMD and as mentioned before is in agreement with *in vitro* studies suggesting a role for STAT3 in proliferation.

Table 2.7 STAT3 signalling in response to acute exercise

First Auth, date	Intervention protocol	Subjects	Muscle	Hypothesis	peak	Main findings
Trennerry 2007	Resistance exercise (3sets of 12 reps max leg extention)	healthy men (n=7)	VL	Intramuscular STAT signalling in response to resistance exercise	2h	STAT3 important in skeletal muscle remodelling and adaptation
Boonsong 2007	90min one legged cycling	healthy men (n=8)	VL	effects of exercise on basal and insulin-mediated changes in the activation (phosphorylation) of the signalling molecules involved in the regulation of SREBP-1c keletal muscle	no change	exercise-induced changes inmuscle SREBP-1c expression might be mediated by the activation of the ERK1/2 pathway
Trennerry 2008	Resistance exercise	healthy young (n=10) vs old (n=10) men	VL	effect of intense resistance ex on STAT activity in aged muscle	2h	enhanced STAT3 signaling responsiveness to proinflammatory factors may impact on mechanisms of muscle repair and regeneration
McKay 2009	300 muscle lengthening contractions	healthy young male (n=12)	VL	Role of IL-6 in satellite cell response to exercise	4h	IL-6 induced STAT3 signalling occur is associated with satellite cell proliferation
Olmedillas 2010	chronic muscle loading (tennis)	professional male tennis players (n=9)	MTB	effect of chronic muscle loading on leptin signalling in human skeletal muscle	n.a	p-STAT3 reduced in non-dominant arm; role for leptin signalling in hypertrophy
Trennerry 2011	12 weeks of resistance training	healthy young men	VL	responsiveness of IL-6 and PDGF-BB to intense exercise, along with STAT3 activation, before and after 12 weeks of resistance training	3h	Unaltered by 12 weeks of training
Toth 2011	300 muscle lengthening contractions	healthy young male (n=12)	VL	STAT3 mediated satellite cell response	24h	IL-6 induced STAT3 signaling occurred exclusively in the nuclei of SCs in response to MLC. SCs were induced to proliferate under the control of STAT3 signaling
Drummond 2011	Single bout resistance exercise	healthy young (n=13) vs old (n=13)	VL	Amino acid transporters and muscle protein anabolism in the elderly	3h	In older adults, the increased nuclear p-STAT3 may be indicative of an stress response to export amino acids from muscle cells.
Guerra 2011	30s Wingate	healthy young men (n=15)	VL	effect of glucose on sprint exercise signaling cascades linked to leptin actions	30min	sprint exercise performed under fasting conditions is a leptin signaling mimetic in human skeletal muscle
Caldow 2012	None	young vs old healthy men	VL	Intramuscular inflammatory gene expression increase with age	resting / fasting	No difference in muscle inflammatory gene expression between young and old men at rest
Fuentes 2012	30s Wingate	healthy male (n=10) vs female (n=17)	VL	Sex dimorphism in skeletal muscle signalling in response to sprint exercise	2h	No difference in skeletal muscle signalling response between men and women

Footnote: Table include all studies investigating intramuscular STAT3 signalling following exercise intervention in human subjects. **Abbr**: h- hour, IL- interleukin, JNK- janus kinase, MLC- muscle lengthening contractions, MTB- muscularis triceps

CHAPTER 3: HYPOTHESES AND OBJECTIVES

Studies 1 and 2: *High intensity intermittent downhill running: a model for testing sensitivity to inflammation and regenerative capacity of skeletal muscle*

We hypothesized that:

- Divergent individual responses to unaccustomed high intensity downhill running might explain the variability observed in circulating markers of muscle damage and that these response patterns are at least in part influenced by the systemic cytokine response.

- Certain individuals are prone to developing sustained inflammation following unaccustomed exercise leading to a second phase of muscle damage that is not directly related to the initial mechanical insult.

- An early anti-inflammatory response is associated with earlier resolution of indirect biomarkers of muscle damage and satellite cell activation.

- The systemic cytokine response following downhill running might induce the mobilization of hematopoietic progenitor cells (HPC) in addition to leukocytosis.

We therefore evaluated:

- Changes in indirect markers of muscle damage following unaccustomed high intensity downhill running over a period of 7 days in healthy young men in an attempt to identify different individual response patterns.

    - *Parameters:* serum creatine kinase (CK) activity, serum myoglobin (Mb) concentration, fatigue and delayed onset muscle soreness (DOMS)

- Pro-inflammatory and regenerative responses following downhill running to determine whether or not an early anti-inflammatory response is associated with earlier resolution of muscle damage and regeneration.

- *Parameters:* differential white blood cell count (diff), serum tumour necrosis factor α (TNFα), serum interleukin-1β (IL-1β), serum interleukin-6 (IL-6), serum interleukin-10 (IL-10), serum granulocyte colony stimulating factor (G-CSF), intramuscular myeloperoxidase (MPO), intramuscular NFκB signalling, satellite cell activity *(Pax7, myoD, myogenin)*, intramuscular (STAT3) signalling.

- Changes in the percentage of circulating hematopoietic progenitor cells in the granulocyte, monocyte and lymphocyte whole blood subpopulations.

  - *Parameters:* CD34 *(that do not express any lineage specific markers such as HLDA-DR, CD33 and CD38).*

Study 3: *Relationship between secondary skeletal muscle damage, leukocytosis and the cytokine response to high intensity intermittent downhill running.*

We hypothesized that:

- Activation status of circulating neutrophils might be related to changes in serum IL-6 concentrations in healthy young men following intermittent high intensity downhill running.

- Greater neutrophil functional activity during the early recovery phase might lead to the development of secondary skeletal muscle damage.

- Peripheral blood mononuclear cell (PBMC) NFκB signalling might be involved in the production of IL-6 following the cessation of exercise.

We therefore evaluated:

- Neutrophil and macrophage activation and their potential association with a second phase of skeletal muscle damage.

  - *Parameters:* neutrophil surface expression of CD11b, C5aR, macrophage surface expression of CD68, CD163 and serum soluble intercellular adhesion

molecule-1 (sICAM-1), sP-selectin, MPO, matrix metalloprotease-9 (MMP-9), CK.

- The pro- versus anti- inflammatory cytokine response in relation to individual changes in circulating indices of muscle damage and PBMC NFκB p65 nuclear localization.

    - *Parameters:* Serum IL-6, soluble interleukin-6 receptor (sIL-6R), interleukin-8 (IL-8) , interleukin-1 receptor antagonist (IL-1ra), interleukin-4 (IL-4) and IL-10.

CHAPTER 4 : METHODOLOGY

This research project involved three human longitudinal non-cross over downhill running intervention studies entitled: " ***NFκB signalling pathway responses to eccentric exercise and its involvement in a second phase of muscle damage*** ". The original research proposal as well as all subsequent amendments has been approved by the Health Research Ethics Committee *(Ethical Reference no: N10/05/154)* at Stellenbosch University.

The Health Research Ethics Committee complies with the South African National Health Act No. 61 2003 as it pertains to health research and the United States Code of Federal Regulation Title 45 Part 46. This research study were therefore conducted according to the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the guidelines for Ethical Research: Principles Structures and Processes 2004 *(Department of Health, South Africa)*.

4.1. Subject recruitment

Inclusion criteria:

- Male between the ages of 18 – 25 years
- Moderate exercise between 2 and 6 hours per week
- Not participating in any hiking or downhill walking
- Informed consent was obtained.

Exclusion criteria:

- Treated for or have a medical history of a chronic inflammatory condition.
- Acute use of anti-inflammatory treatment within 3 weeks prior to study.
- Use of any prescribed or over the counter medication/ supplements.
- Any form of clinical infection
- Skeletal muscle or exercise related injury in the previous 3 months.
- Informed consent was not obtained.

Volunteers were recruited through flyers *(Appendix A)* from the student population at Stellenbosch University. A total of sixty seven healthy young men volunteered to participate in the research project. From these volunteers nine did not fit the inclusion criteria and were therefore excluded. Since participation was totally voluntary and subjects had the right to withdraw at any time, a further five volunteers withdrew for personal reasons. A total of fifty three healthy male volunteers were thus included in the research project and informed of the study procedure verbally and in writing before signing an informed consent form *(Appendix B)*.

These 53 participants were randomly subdivided into a downhill run (DHR) (n=40) and control group (n=13). Preliminary research done by our group showed different individual creatine kinase (CK) response patterns following intermittent downhill running *(60 min, 10% decline @ 85% VO_2max)* (van de Vyver *et al.* 2012). In the current research project, higher numbers are therefore necessary in the experimental group in order to maintain statistical power while investigating these individual differences in the time course and magnitude of CK appearance and resolution in circulation.

Table 4.1 Subject characteristics

	Age (yr)	**Height** (m)	**Weight** (kg)	**BMI** ($kg.m^{-2}$)	**VO_2max** ($ml.min.kg^{-1}$)
(n=53)	20.4 ± 0.3	1.81 ± 0.1	76.7 ± 2.1	23.8 ± 0.4	50.7 ± 1.1

Footnote: Values are presented as mean ± SE. BMI was calculated using the following formula: body weight (kg)/ $height^2$ (m^2). VO_2max was determined using an incremental test to fatigue *(refer to section 4.2.1.1)*. **Abbr:** BMI – Body Mass Index; kg- kilograms; m- meter; ml – millilitre; yr –years.

4.2 Study design

This research project was conducted in the Exercise Physiology laboratory, Department of Physiological Sciences, Stellenbosch University.

Participants were instructed to refrain from using any anti-inflammatory drugs, painkillers, immune boosting supplements or alcohol for the duration of the study. Furthermore, participants were encouraged to limit their physical activity and to keep a diary of any

unaccustomed activities *(Appendix C)* starting one week before the baseline testing and continue throughout the duration of the study. At the end of the study, participants reported any unaccustomed physical activity for the previous 3 weeks. This was necessary to have some control over the daily variation in indirect markers of muscle damage and satellite cell activation status.

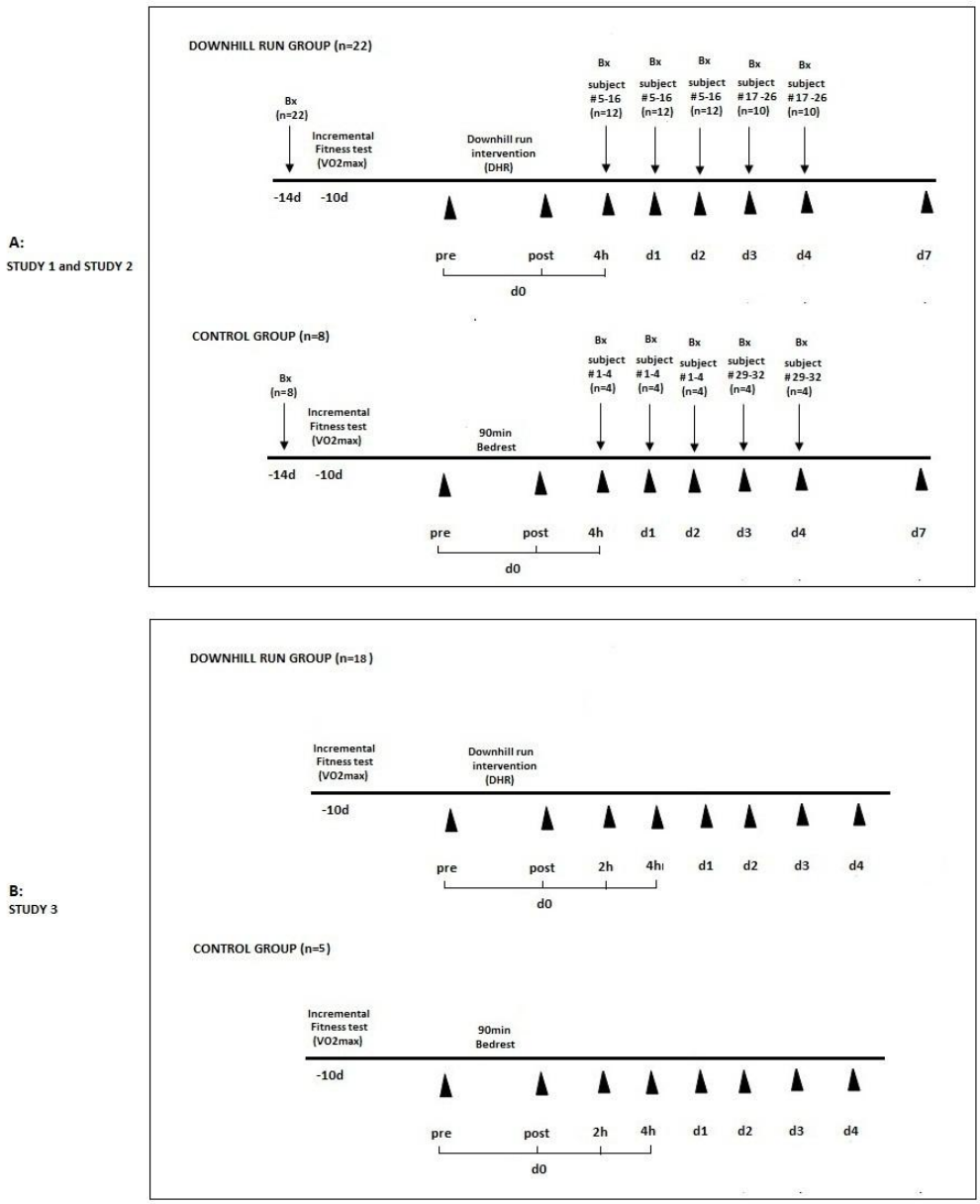

Figure 4.1 Study design A – Study 1 and Study 2 **B-** Study 3. **Abbr:** Bx –muscle biopsy (*vastuls lateralis)*, d – day, VO_2max – fitness test, DHR – downhill run, ▲ blood sample

4.2.1 Exercise testing

All participants were familiarized with the treadmill *(Runrace, Technogym, Italy)* and the exercise testing procedures explained to them verbally, upon their first arrival in the exercise laboratory.

In *study 1* and *study 2*, both groups performed a fitness *(VO_2max)* test (*refer to section 4.2.1.1, below*) 2-4 days after the baseline muscle biopsy. This was necessary to deter any influence that exercise testing might have on satellite cell activity. Since the biopsy procedure is invasive, participants were given sufficient time to recover, before they participated in the incremental fitness test. In *study 3 (no muscle biopsies)*, both groups performed the fitness test approximately 10 days before the intervention protocol on day 0. In addition to the fitness test, the DHR group also participated in a downhill running intervention protocol (*refer to section 4.2.1.2, p. 71*), while the control group were subjected to bed rest for the same duration as the intervention protocol.

4.2.1.1 Incremental fitness test (VO_2max)

Following a 5 minute (min) warm up on the treadmill *(RUNRACE HC1200; Technogym)* the incremental fitness test started at a standard speed of 8 $km.h^{-1}$ at a level gradient. Thereafter, the speed was increased every 30 seconds by 0.5 $km.h^{-1}$ until voluntary exhaustion. Participants were encouraged verbally to continue for as long as possible although each participant could stop the test at any time due to fatigue or inability to continue. The peak treadmill speed (PTS) as well as the total time duration of the test were recorded together with breath-by-breath gas analysis data.

Breath-by-Breath gas analysis: Before every incremental exercise test the metabolic system *(Oxycon Pro, Jaeger, Germany)* was calibrated with atmospheric O_2 (20.93%) and a known concentration of CO_2 (4.97%). Following calibration the system was linked to the oxygen mask worn by the participants throughout the incremental test. Each participants VO_2max was calculated using the highest average volume of oxygen consumed over a period of 30 seconds that coincided with an respiratory exchange ratio (RER) above 1.1 .

4.2.1.2 Downhill run

Participants in the downhill run (DHR) group performed a 60-min intermittent downhill run protocol consisting of 12 x 5 min bouts against a 10% decline at a constant velocity of 15 $km.h^{-1}$ on a motorized treadmill *(RUNRACE HC1200; Technogym*). In between each bout of downhill running, participants were allowed a 5 min standing rest and voluntary fluid *(water)* intake.

4.2.2 Fatigue and muscle soreness assessment

Participants were asked to self-asses the fatigue and muscle soreness they experience by answering a questionnaire based on a modified Borg scale *(Appendix D)*. All of the participants completed the questionnaire before the downhill run as well as immediately, 2 hours (h) (*study 3 only*), 4h and on days 1, 2 , 3, 4 *(all of the studies)* and 7 (*study 1 and study 2*) post intervention. The first section of the questionnaire focusses on fatigue in different muscle groups (*quadriceps, hamstrings, calves*) of the lower limb, whereas the second part of the questionnaire focusses on muscle soreness specifically in the *quadriceps* muscle group.

4.2.3 Sample collection

Throughout the research project, both the downhill run (DHR) and control groups were subjected to the exact same study design with regards to sample collection. In *study 1* and *study 2*, eight venous blood samples and four skeletal muscle biopsies *(vastus lateralis)* were collected from both groups over a period of 21 days. In *study 3*, eight venous blood samples were collected from both groups over a period of 5 days without the collection of any skeletal muscle biopsies.

4.2.3.1 Blood sampling

A qualified phlebotomist collected blood samples from the forearm vein in the supine position using the venipuncture technique in combination with the BD vaccutainer system.

On the day of the intervention protocol (day 0) a sample was collected pre- (*all of the studies*), immediately post (*all of the studies*), 2h post *(study 3 only)* and 4h post-(*all of the studies*) the downhill run for the DHR group and pre (*all of the studies*), immediately post (*all of the studies*), 2h post *(study 3 only)* and 4h (*all of the studies*) post a 90 min bed rest for the control group. On days 1, 2, 3, 4 (*all of the studies*) and 7 (*study 1 and 2 only*) one sample was collected every day *(between 12:00 and 14:00)* from each participant *(refer to Fig. 4.1, p. 69)*. At each time point a standard Serum Separating Tube (SST) as well as a EDTA tube *(BD Vacutainer SST II Advance, Becton Dickinson and Company)* was filled with blood and immediately processed for either storage or analysis.

- SST tubes were left to coagulate at room temperature for no longer than 10 minutes and then centrifuged at 3000 RPM for 10 min @ 4 °C. Serum was immediately frozen in aliquots and stored at - 80 °C. The serum samples were analysed *(refer to section 4.3.1, p. 75)* for indirect markers of muscle damage *(creatine kinase, myoglobin)*, a metabolic stress marker *(cortisol)* and for various cytokines and adhesion molecules (*tumour necrosis factor- alpha (TNFα),interleukin-1 receptor antagonist (IL-1ra), interleukin 1beta (IL-1β), interleukin-4 (IL-4) interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), granulocyte colony stimulating factor (G-CSF), macrophage inflammatory protein-1beta (MIP-1β), soluble intracellular adhesion molecule-1 (sICAM-1), vascular cellular adhesion molecule-1 (VCAM-1), soluble P-Selectin (sP-Selectin) soluble IL-6 receptor (sIL-6R), metalloproteinase-9 (MMP-9), myeloperoxidase (MPO)).*

The whole blood samples collected in the EDTA tubes were immediately processed and analyzed. Absolute and differential white blood cell count was determined using a universal cell counter *(Cell-Dyn 3700, Diagnostech)* (*study 1 and study 3*) *(refer to section 4.3.2.1, p. 79).* Whole blood was analysed using multicolour flow cytometry to determine the expression of markers indicatve of neutrophil and macrophage activation status *(CD11b, CD163, CD68, CD88, intracellular MPO, CD45)* (*study 3*) and circulating haematopoietic progenitor cells *(CD34, CD38, CD33, HLA-DR)* (*study 2*) *(refer to section 4.3.2.2, p. 79).* Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood and stained *ex vivo* using immunofluorescent antibodies to determine nuclear *vs* cytoplasmic localization of the NFκB p65 transcription factor *(refer to section 4.3.3, p. 83)*.

4.2.3.2 Needle Muscle biopsies

A qualified medical doctor (Mb.ChB) who is well trained in the procedure performed the biopsies using a sterile 5mm Bergstrom muscle biopsy needle *(Dixons Surgical Instruments Ltd. SS11 8YB-UK)* with assisted manual suction (Bergstrom 1975, Hennessey *et al.* 1997). Local analgesia *(Xylotox L – 1.8ml Lignocaine HCl/ 2% m/v Noradrenaline 1:80000 and Xylotox S.E Plain 1.8 ml Lignocaine HCl, Adcock Ingram Ltd.)* was injected into the biopsy site *(skin and fascia)* and as is customary for the procedure, no sedatives were provided.

In *study 1* and *study 2*, all of the participants in both the control (n=8) and DHR (n=22) groups were subjected to a baseline muscle biopsy from the *vastus lateralis* muscle of the left leg 14 days prior to the intervention protocol. In the early recovery phase, three biopsies were taken from four participants in the control and twelve participants in the DHR group at 4h , day (d) 1 and d2 . In the late recovery phase on d3 and d4 biopsies were taken from the remaining four participants in the control group and ten participants in the DHR group *(refer to Fig. 4.1 p. 69 and Fig. 4.2 p. 74)*. Subjects were randomly selected for the collection of biopsies during either the early (4h, d1, d2) or late recovery phase (d3, d4).

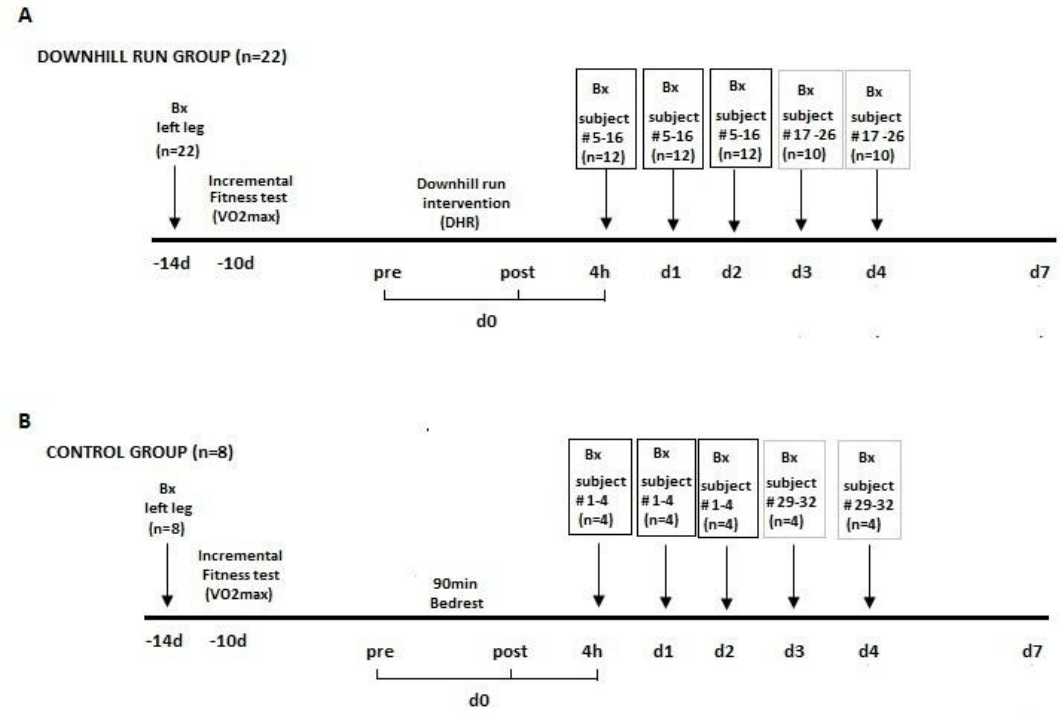

Fig 4.2 Timeline for skeletal muscle biopsy sampling. Panel A – DHR group **Panel B -** Control group. There was a distance of 3cm between biopsy sites on the same leg. **Abbr:** Bx – muscle biopsy, d – day, h - hour.

Approximately 50-100 mg muscle was collected at each time point and divided into three parts:

- One piece of each biopsy was snap frozen in liquid nitrogen and stored at -80 °C. This was used for Western blot analysis *(refer to section 4.3.4, p. 84)* to determine intramuscular signalling by assessing the ratio between total and phoshorylated protein levels of the following transcription factors: signal transducer and activator of transcription-3 (STAT3), Nuclear factor kappa-B inhibitor protein (IkBα) and P38 mitogen activated protein kinase (P38 MAPK). Western blot analysis was further used to assess myoD and myogenin protein levels as an indication of satellite cell activity. Intramuscular neutrophil and macrophage activity was assessed by detecting changes in myeloperoxidase (MPO) protein levels.

- A second piece of each biopsy was embedded in tissue freezing medium *(Jung #020108926)* and frozen in iso-pentane that had been pre-cooled in liquid nitrogen. The sample was stored at -80°C until cryosectioning between -20 °C and -18 °C using a microtome (*CM1100, Leica)*. Each individual participant's fibre type composition was determined using a myosin ATPase assay at a pH level of 4.3 in order to distinguish between Type I and Type II fibres *(refer to section 4.3.7, p. 88)*. Cryosections were also stained with immunofluorescent antibodies against Pax7 *(marker of quiescent and proliferating satellite cells)* and laminin *(basil lamina marker) (refer to section 4.3.8, p. 89).*

- The third part of the biopsy was placed in a 3-4% paraformaldehyde solution containing 2.5% sucrose. These samples were embedded in LR-White Resin for electron microscopy to visualize any structural signs of muscle damage *(z-line streaming/ sarcolemmal damage) (refer to section 4.3.9, p. 90)*.

4.3 Laboratory analysis

Laboratory analysis was performed in the following laboratories: molecular and histology laboratories of the department of Physiological Sciences (Stellenbosch University), the Central Analytical Facility at Stellenbosch University (live cell imaging and flowcytometry unit, DNA sequencing unit), BD BioSciences training centre (Tygerberg campus, Stellenbosch University), the national health laboratories (Electron microscopy unit, Tygerberg hospital), Pathcare commercial pathology laboratory (Stellenbosch, South Africa).

4.3.1 Analysis of serum

4.3.1.1 One step sandwich assays: indirect markers of muscle damage

For both groups the serum samples for all time points were analyzed by a commercial pathology laboratory *(Pathcare)* using one-step sandwich assays for creatine kinase (CK) activity *(CARDIAC Calibrator # 386371)* and myoglobin (Mb) concentration *(CARDIAC Calibrator # 973243)*. The normal clinical reference range for CK was between 15 and 195 $u.L^{-1}$ at 37 °C and for Mb between 17 and 106 $ng.mL^{-1}$.

4.3.1.2 Bio-Plex Pro Human Cytokine assays (study 1 and study 2)

Serum samples were analysed for various cytokines *(TNFα, IL-6, IL-1β, IL-10 G-CSF)*, chemokines (MIP-1β) and adhesion molecules *(ICAM-1, VCAM-1)* using either the Bio-Plex Pro Human cytokine Group I assay *(6-Plex, #M500002ALX, BioRad Laboratories)* or the Bio-Plex Pro Human cytokine Group II assay *(2-Plex, #MF000000AY, BioRad Laboratories)* in combination with the Bio-Plex suspension array system *(Luminex 100)*.

These assays are magnetic bead-based multiplex assays using an antibody directed against the desired targets that is covalently coupled to internally dyed beads. The coupled beads then react with the serum sample, while a series of washing steps remove all unbound protein. A biotinylated detection antibody specific to an epitope that is different from that of the capture antibody is then added resulting in the formation of a sandwich around the target molecules. Streptavidin-phycoerythrin *(streptavidin-PE)* is added and binds to the biotinylated detection antibodies on the beads. The data were acquired using a dual-laser, flow-based microplate

reader *(Luminex 100)* that detects the internal fluorescence of the individual dyed beads as well as the fluorescent reporter signal on the bead surface. The intensity of fluorescence detected on the beads indicated the relative quantity of target molecules in each sample. The data were presented as fluorescent intensity on the Bio-Plex Manager™ software. The observed concentration of each target molecule was calculated from its fluorescent intensity based on the 5PL regression standard curve generated for each target.

Table 4.2 Normal physiological ranges of group I cytokines

	pg/ml			
Analyte	Concentrations in Range	Observed Concentration	Median of Concentrations in Range (n=66)	Mean of Concentrations in Range (n=66)
Group I Cytokine Assays				
G-CSF	<1.50	<1.50	0.00	0.02
IL-1β	<0.70	0.02–0.70	0.00	0.01
IL-6	0.50–9.00	0.02–9.00	0.00	0.73
IL-10	0.40–2.00	0.10–2.00	0.00	0.13
MIP-1β	5.00–47.00	1.70–47.00	11.24	14.75
TNF-α	6.00–98.00	0.10–98.00	0.00	5.92

Footnote: Information provided by BioRad Laboratories. *Bio-Plex suspension array system tech note 6029*.

Samples were prepared according to the manufacturer's instructions. For the Bio-Plex Pro Human cytokine Group I assay *(6-Plex, #M500002ALX, BioRad Laboratories),* one volume of serum was diluted with 3 volumes of sample diluent (*Item #10014641*). For the Bio-Plex Pro Human cytokine Group II assay *(2-Plex, #MF000000AY, BioRad Laboratories)* serum was diluted 1:4 in sample diluents and then further diluted 1:25 using standard diluent (*L9703888 Rev C*). A series of 8 standards each with a specific known concentration of all the target molecules was prepared according to the manufacturer's instructions using a lyophilized standard (*10001475 Rev B*) and standard diluent (*L9703888 Rev C*).

The standards and each serum sample was assigned to a specific well of the 96-well microplate. The total number of wells that were used including 25% excess were determined and the volume of the coupled beads , detection antibodies and Streptavidin-PE needed were calculated accordingly. All the solutions and buffers used during this assay was obtained

from the Bio-Plex Pro Human cytokine Group I and II assay kits respectively. All standards were run in duplicate with mean covalent of variance and detection limit for each target molecule: IL-1β *(13.4% CV, 0.50 pg.mL^{-1})*, IL-6 *(10.86 % CV, 0.50 pg.mL^{-1})*, IL-10 *(10.3 % CV, 1.5 pg.mL^{-1})*, G-CSF *(10.3% CV, 0.8 pg.mL^{-1})*, MIP-1β, *(14.1% CV, 1.3 ng.mL^{-1})*.

For step by step Bio-plex pro human cytokine assay procedure refer to *Appendix E.1.*

4.3.1.3 Enzyme-linked immunosorbent assay: Cortisol (study 1)

Serum cortisol concentrations were determined using a solid phase enzyme-linked immunosorbent assay (ELISA) (*EIA 1887 DRG Cortisol ELISA, # 43K041*). The assay is based on the principle of competitive binding, where the cortisol in the serum samples compete with a cortisol-horseradish peroxidise conjugate for binding with a monoclonal antibody directed against an antigenic site on the cortisol molecule. The amount of bound peroxidase conjugate is inversely proportional to the concentration of cortisol in each sample. All solutions and buffers used in this assay were part of the ELISA kit (*EIA 1887 DRG Cortisol ELISA, # 43K041*) and prepared as per manufacturer's instructions. The cortisol concentration of each serum sample were read directly from the standard curve.

For step by step ELISA procedure refer to *Appendix E.2.*

4.3.1.4 Milliplex xMAP immunoassays (study 3)

All serum samples collected from the downhill running participants in study 3 were analysed for various cytokines *(IL-1ra, IL-4, IL-6, IL-8, IL-10) (Milliplex human cytokine/chemokines panel I HCYTOMAG-60K-05)*, soluble IL-6 receptor *(sIL-6R) (Milliplex human soluble cytokine receptor HSCR-32K-01),* soluble ICAM-1, P-selectin, MPO *(Milliplex Human CVD panel 2, HCVD2MAG-67K-03)* and MMP-9 *(Milliplex human MMP2 kit, HMMP2-55K-01*) using Milliplex map immunoassay kits in combination with the Luminex 100 suspension array system.

These assays are based on the Luminex xMAP technology that used proprietary techniques to internally color code microspheres with fluorescent dyes. A distinctly colored bead coated with a specific capture antibody captures the analyte of interest in the serum sample. A biotinylated detection antibody is then introduced and incubated with Streptavidin PE conjugate. The beads are then passed

rapidly through a laser, which excites the internal dyes, a second laser excites the fluorescent dye *(PE)* on Streptravidin. Based on this fluorescent reporter signal the results of each individual bead is quantified.

Samples were prepared according to the manufacturer's instructions. For the Milliplex human cytokine/chemokines magnetic bead panel *(5-Plex, #HCYTOMAG-60K, Milliplex xMAP kit, #2058418)* undiluted serum was used in the assay. For the Milliplex human MMP2 panel *(#HMMP2-55K, Milliplex xMAP kit, #2058420),* one volume of serum was diluted with 20 volumes of assay buffer (*Item #2026205*). For the Milliplex human soluble cytokine receptor panel serum was diluted with 5 volumes of serum matrix and for the Milliplex human cardiovascular panel 2 *(#HCVD2MAG-67K, Milliplex xMAP kit, 32060448)* serum was diluted with 100 volumes of serum matrix. A series of 7 standards each with a specific known concentration of all the target molecules was prepared according to the manufacturer's instructions using a lyophilized standards and standard diluent provided in each kit respectively. In addition, two quality control samples *(also provided in each kit respectively)* were run in duplicate to ensure the accuracy of each assay.

All standards were run in duplicate with mean covalent of variance and detection limit for each target molecule: IL-1ra *(2.1% CV, 8.3 pg.mL^{-1})*, IL-4 *(2.9% CV, 4.5 pg.mL^{-1})*, IL-6 *(2% CV, 0.9 pg.mL^{-1})*, IL-8 *(1.9% CV, 0.4 pg.mL^{-1})*, IL-10 *(1.6% CV, 1.1 pg.mL^{-1})*, sIL-6R *(5.6% CV, 3.6 pg.mL^{-1})*, MMP-9 *(7% CV, 1 pg.mL^{-1})*, sP-Selectin *(15% CV, 0.051 pg.mL^{-1})*, sICAM-1 *(15% CV, 0.019 pg.mL^{-1})*, MPO *(15% CV, 0.036 pg.mL^{-1})*.

For the step by step immunoassay procedures refer to *Appendix E.3*

4.3.2 Analysis of whole blood

4.3.2.1 Absolute cell counts

Whole blood samples *(EDTA tubes)* for both groups were analysed using an automated hematology cell counter (*Cell-Dyn 3700, Abbott Diagnostics).* Absolute red blood cell (RBC) count as well as total (WBC) and differential white blood cell (diff) count were determined for all time points.

4.3.2.2 Multicolour flow cytometric analysis

4.3.2.2.1 Circulating haematopoietic progenitor cells (study2)

Whole blood collected in EDTA tubes immediately before and 4h post downhill running were analysed for the expression of the CD34 labelled fluorescein isothiocyanate *(Mouse Anti-human, #560942)*, CD38 labelled phycoerythrin *(Mouse Anti-human, # 560981)*, CD33 labelled allophycocyanin *(Mouse Anti-human , #551378)*, and HLA-DR labelled peridin chlorophyll protein *(Mouse Anti-human, #347402)* antigens (*Becton Dickinson*) by using four colour staining. Since it is a multicolour cytometric analysis fluorescent compensation settings were established through an compensation experiment using BD anti-mouse compensation beads (*refer to Appendix E.4.1*).

Following the compensation experiment, whole blood samples were immunofluorescence labelled by multicolour staining *(refer to Appendix E.4.2)* and analyzed using flow cytometry *(FACSCaliur system , Becton Dickinson)*. Analysis was performed by using separate gates on the lymphocyte, monocyte and granulocytes regions with a minimum of 50 000 events acquired (*refer to Fig. 4.3 p. 80*). The percentage of cells expressing each of the antigens were determined in all of the regions using FACSDiva software (*Becton Dickinson)*.

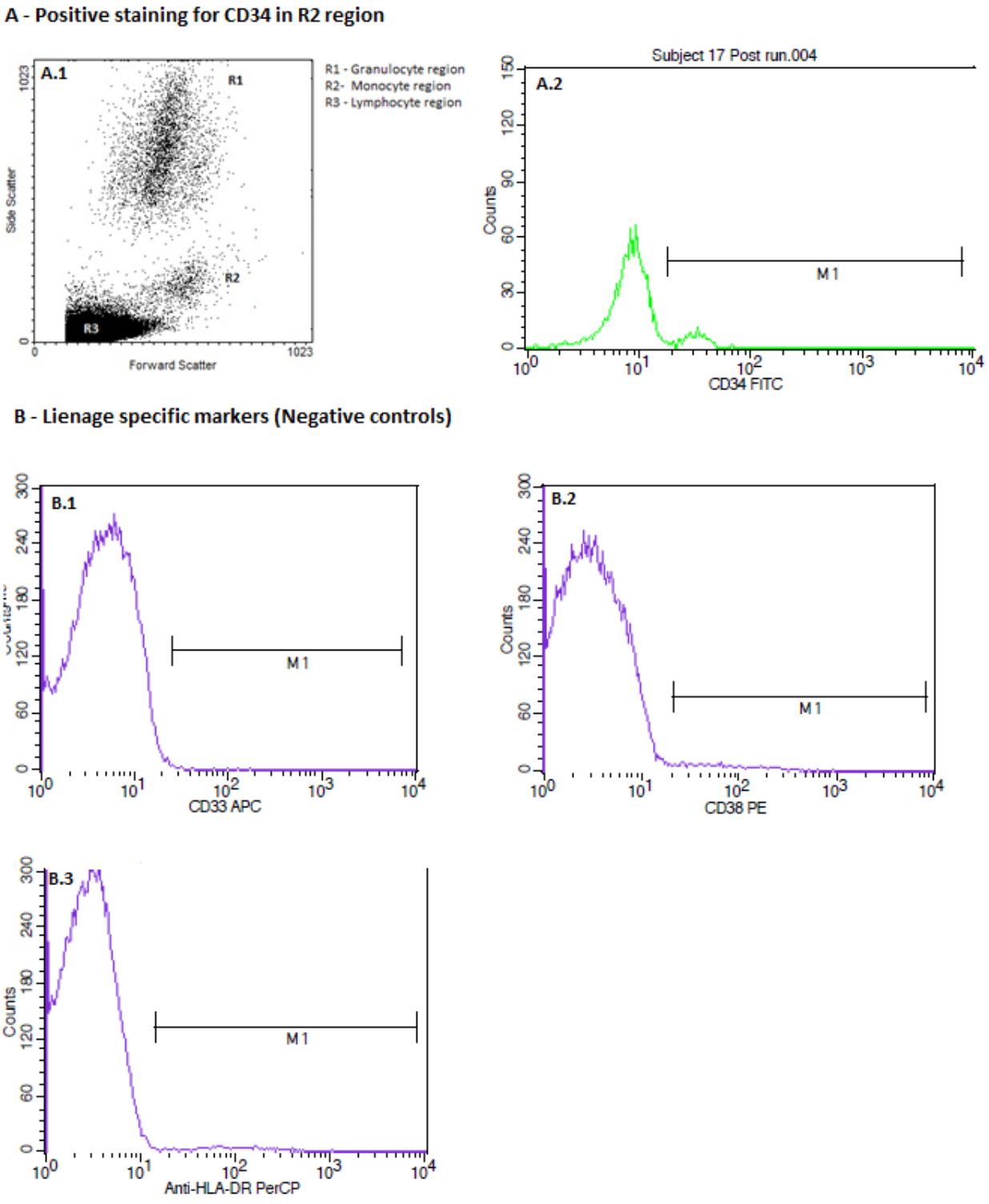

Fig. 4.3 Multicolour flow cytometric analysis. A.1 Whole blood subpopulations**. A.2** $CD34^{+}$ **B.1** $CD33^{-}$. **B.2** $CD38^{-}$ **B.3** $HLA\text{-}DR^{-}$ haematopoietic progenitors detected in the monocyte region (R2).

4.3.2.2.2 Activation status of circulating neutrophils and macrophages (study 3)

Whole blood *(EDTA tubes)* collected during *study 3* immediately before , immediately post, 2h post and 4h post downhill running were analysed using multicolour fluorescent staining in combination with flowcytometry (*FACS Canto, BD Biosciences*).

The expression of intracellular myeloperoxidase (MPO) *(FITC Anti-human, #333138, BD Biosciences)*, CD11b/Mac1 *(Horizon V450 Anti-human, # 560480, BD Biosciences)*, CD163 *(APC Anti-human , #333610, Biolegend)*, CD88/C5aR *(PE Anti-human #344304, Biolegend)* and CD68 *(PerCPCy5.5 Anti-human, #333814, Biolegend)* antigens were determined within the granulocyte and monocyte region respectively by setting polygonal gates surrounding these specific populations of cells. CD45 *(APCCy7 Anti-human, #557833, BD Biosciences)* was used as a lymphocyte control marker to ensure that no lymphocytes were included in the monocyte gate.

A minimum of 10 000 events were recorded in the granulocyte region prior to data analysis. Since it is a multicolour cytometric analysis fluorescent compensation settings were established through an compensation experiment *(refer to Appendix E.4.3)* and regions of positive and negative staining were determined through a FMO *(fluorochrome minus one)* experiment *(refer to Appendix E.4.4)* using BD anti-mouse compensation beads (*BD comp bead plus #51-9006274, BD comp bead negative #51-9006227*). Whole blood samples were immunofluorescently labelled by multicolour staining *(refer to Appendix E.4.5).*

Data analysis was performed by using separate gates on the monocyte and granulocyte regions with a total of 10 000 events acquired in the granulocyte region. The percentage of cells expressing each of the antigens as well as the co-expression of antigens were determined in each of the regions using FACSDiva software *(BD Biosciences)*.

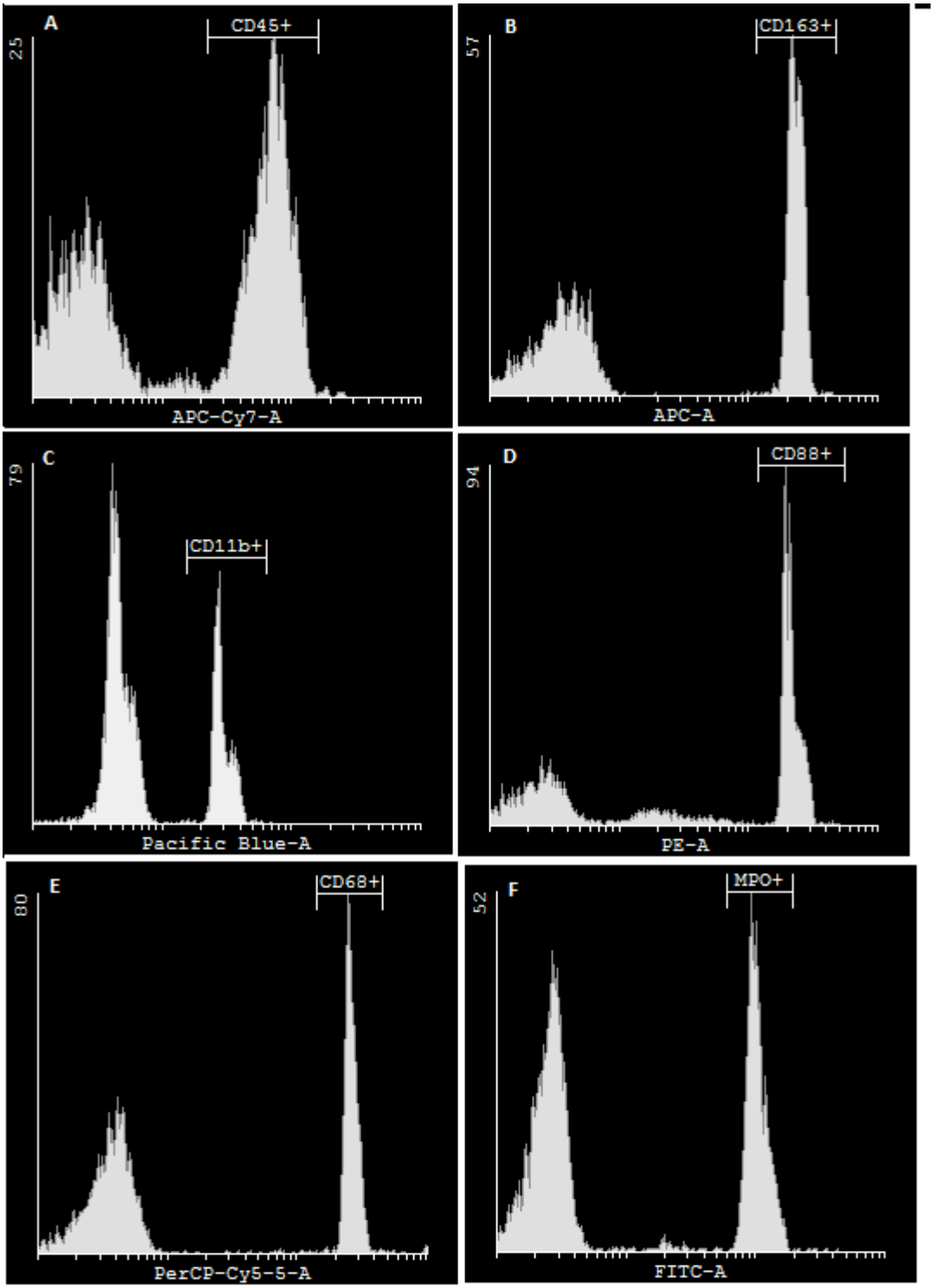

Fig. 4.4 Multicolour flow cytometric analysis. A. APCCy7 (CD45) **B.** APC (CD163) **C.** Pacific blue/ V450 (CD11b) **D.** PE (CD88) **E.** PerCPCy5.5 (CD68) **F.** FITC (MPO) areas of positive staining**.**

4.3.3 Analysis of Peripheral Blood Mononuclear Cell (PBMC)

Peripheral mononuclear cells were isolated from whole blood collected in EDTA tubes on d0 *(study 3).* Following collection all samples reached room temperature before isolation to ensure optimum recovery of PBMCs. For step by step isolation procedure refer to *Appendix E.5*. Following isolation of PBMCs the samples collected at 2h following downhill running (*study3*) were immediately stained using fluorescent antibodies in order to determine nuclear vs cytoplasmic localization of the NFκB p65 subunit. For step by step immunofluorescent staining procedure refer to *Appendix E.5.1*.

Fluorescent microscopy was performed on the Olympus IX81 inverted fluorescent microscope and images taken at x40 magnification *(Olympus LUCPlanFLN 40x/0.60 Ph2 ∞/0-2 FN22)* using F-view-II cooled CCD camera *(Soft Imaging systems)*. The percentage co-localization of nuclei *(4'6-diamidino-2phenylindale, DAPI) (blue)* and NFκB p65 *(TexasRed) (red)* were determined for each cell using ImageJ software (*Image J 1.43, Wayne Rasband, National Institute of Health, USA, rsb.info.nih@gov/ij*). Nuclear localization was determined in an average of 94.6 ±22.6 isolated cells for each participant.

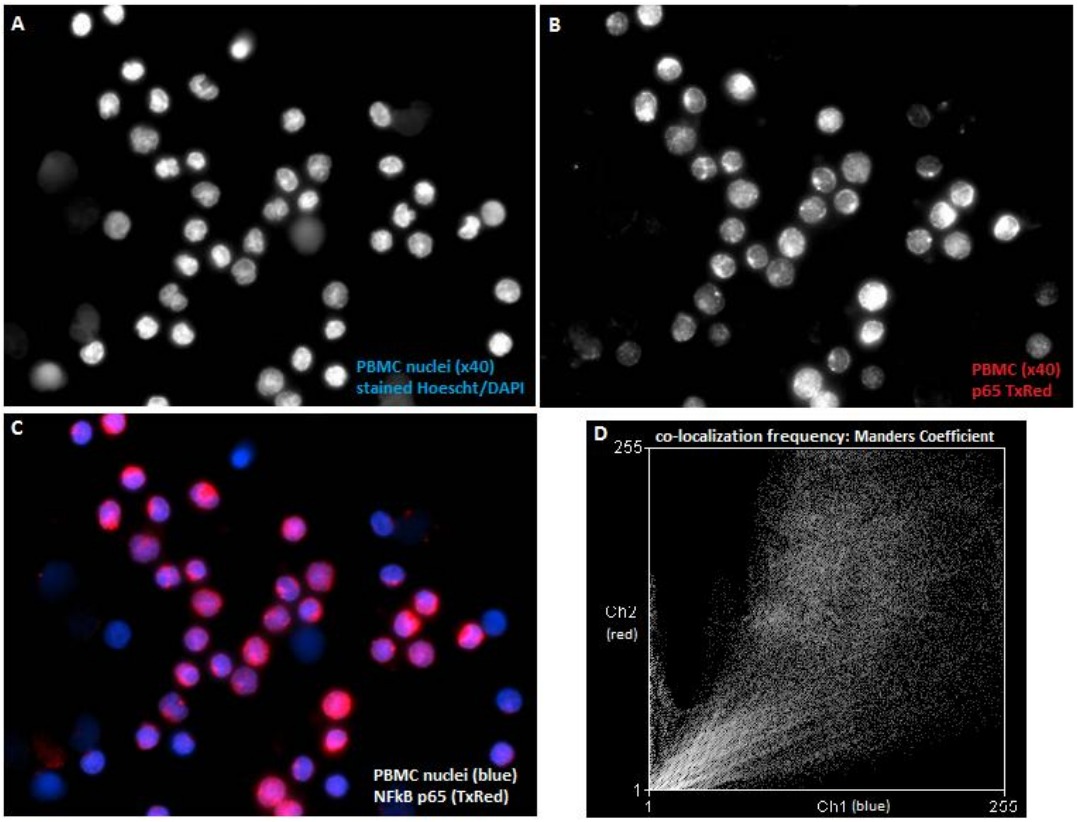

Fig. 4.5 NFκB signalling in PBMCs at 2h following DHR. Panel A – Monocrome image of PBMC's nuclei stained by Hoescht (DAPI). **Panel B –** Monocrome image of intracellular NFκB p65 (TxRed) expression. **Panel C –** RBG image indicating PBMC nuclei (blue) and NFκB p65 (Red) expression **. Panel D –** Co-localization frequency scatterplot. Statistical analysis: Co-localization was determined by ImageJ software and Mander's coefficient reported.

4.3.4 SDS-PAGE and Western blotting

A small piece of snap frozen muscle was sonicated *(VirSonic 300,Virtis Comp Inc. Gardiner N.Y 12525)* together with 100µl RIPA buffer. The samples were kept on ice the entire time to prevent the proteins from denaturing. After sonication the lysates were centrifuged @ 8000 RPM for 10 minutes at 4°C. 5µl of each lysate was immediately used for the Bradford protein quantification method (*refer to Appendix E.6.1*).

For each biopsy, the specific volume of lysate containing a 50 µg protein *(determined using the Bradford protein quantification method)* was added to 8 µl of sample buffer and made up

to a final volume of 35µl using RIPA buffer. Samples were vortexed and proteins denatured at 95°C for 5 min in a heating block before being stored at -80°C. 1.0 mm Acrylamide gels containing 10 wells were used in combination with the BioRad western blotting system for separating the proteins in the extracted samples *(refer to Appendix E.6.2)*. For each gel the first lane was loaded with a standard protein marker *(peqGOLD, protein ladder IV , #37954)* followed by one or two control samples (*refer to table 4.3, p. 86*) in lane 2 and 3, the remaining 7 lanes of each gel were loaded with experimental samples *(different biopsy time points)*. To ensure accurate comparison, samples from a single subject was loaded onto the same gel.

The SDS-page and western blotting analysis were repeated twice for all samples to ensure accurate results. The same sample was loaded in the second lane of each gel that served as a control between different gels. And in addition an antibody against β-actin *(Santa Cruz biotechnology, sc-81178)* was also used to as a control for equal protein loading on each gel. Once developed on film the blots were scanned *(2400dpi, HP psc 1100 series)* and digitized using computer software (*Image J 1.43, Wayne Rasband, National Institute of Health, USA, rsb.info.nih@gov/ij*). The data was then normalized to a percentage value with the control sample on each gel (lane 2) set at 1.

Table 4.3 Antibody panel for western blotting

Primary antibody panel					Control Sample		
	Clone	Specificity	Mw	Dilution		ug protein	Exposure time
MyoD (5.8A) *Santa Cruz, sc-32758*	mouse monoclonal	mouse,human,rat	45kDa	1:500	**Uninjured human muscle** *vastus lateralis*	70 ug	5min
Myogenin (F5D) *Santa Cruz, sc-12731*	mouse monoclonal	mouse,human,rat	36kDa	1:500	**Uninjured human muscle** *vastus lateralis*	70 ug	5min
MPO (AF3174) *R&D systems, WTN01*	goat polyclonal	human	65kDa	1:500	**human isolated neutrophil**	70 ug	1min
pSTAT3 (Y705) *Cell Signalling, #9131S*	rabbit polyclonal	mouse,human,rat	72kDa	1:500	**Uninjured human muscle** *vastus lateralis*	50 ug	1min
STAT3 *Cell Signalling,#9132*	rabbit polyclonal	mouse,human,rat	79-86kDa		**Uninjured human muscle** *vastus lateralis*	50 ug	1min
B-Actin (ACTBC1187) *Santa Cruz, sc-81178*	mouse monoclonal	mouse,human,rat	43kDa	1:1000	**Uninjured human muscle** *vastus lateralis*	50 ug	1min
p-P38 MAPK *Cell Signalling, #9211S*	rabbit polyclonal	mouse,human,rat	43kDa	1:500	**Uninjured human muscle** *vastus lateralis*	50 ug	5min
P38 MAPK *Cell Signalling, #9212*	rabbit polyclonal	mouse,human,rat	43kDa	1:500	**Uninjured human muscle** *vastus lateralis*	50 ug	5min
IkBalpha (H-4)Sc-1643 *Santa Cruz, #C1011*	mouse monoclonal	human	35kDa	1:1000	**Uninjured human muscle** *vastus lateralis*	50 ug	5min
p-IkBalpha (ser32) Sc-7977 *Santa Cruz, #1911*	goat polyclonal	human	41kDa	1:1000	**Uninjured human muscle** *vastus lateralis*	50 ug	5min

Footnote: All antibodies were diluted using TBS-T. **Abbr:** kDa –kilodalton, min- minutes, Mw – molecular weight, ug –microgram.

4.3.5 Bio-Plex Phospho-protein detection assays

The principle behind the Bio-Plex phosphoprotein assays is that an antibody directed against the phosphorylated target proteins *(IkBα and NFκβ p65)* is covalently coupled to internally dyed beads. The coupled beads are then allowed to react with a lysate sample containing the target protein followed by addition of a biotinylated detection antibody resulting in the formation of a sandwich of antibodies around the target protein. Streptavidin-phycoerythrin (Streptavidin-PE) is then added and binds to the biotinylated detection antibody on the bead surface. Data from this reaction is then acquired using the Bio-Plex suspension array system *(Luminex 100)*, a dual-laser, flow-based microplate reader system. The lasers and associated optics detect the internal fluorescence of the individual dyed beads as well as the fluorescent signal on the bead surface. The data are then presented as fluorescence intensity on the Bio-Plex Manager ^{TM} Software. For this specific assay TNFα treated HeLa cell lysate (*L9704215*

Rev E) was used as a positive control and phosphatase treated HeLa cell lysate (*L9704215 Rev E*) was used as control for background fluorescence.

Lysates from the tissue samples were prepared using a Bio-Plex Phoshoprotein detection reagent kit *(#171-304004, BioRad Laboratories)* and cell lysis kit *(#171-304011, BioRad Laboratories)* according to the manufacturer's instructions.

- Tissue sample was rinsed with cell wash buffer and cut into a piece of approximately 3x3 mm.

- 500µl of lysing solution *(40*µl *factor 1, 20*µl *factor 2, 9.9ml cell lysis buffer, 40*µl *500mM PMSF*) was added to the piece of tissue and it was grinded using a tissue grinder (*VirSonic 300,Virtis Comp Inc. Gardiner N.Y 12525*) and immediately frozen at -80 °C.

- Samples were thawed, sonicated *(VirSonic 300,Virtis Comp Inc. Gardiner N.Y 12525*) briefly and centrifuged at 4 500 g's for 20 minutes @ 4 °C. The supernatant was collected and transferred to a new eppendorf.

- The protein concentration of each lysate was determined using the Bradford protein quantification method as previously described *(refer to Appendix E.6.1)*. All samples were diluted to ensure that the protein concentration were the same in each lysate (520 ug.mL^{-1}), and an equal volume of assay buffer were added to the lysate.

- Lysates were stored @ -20 °C.

The experiment *(Bio-Plex Phospho-IkB-α (Ser^{32}/Ser^{36}) assay (#171-V20758) and Phospho-NF-κB p65 (Ser^{536}) assay (#171-V24937))* was planned and each lysate was assigned to a specific well of the 96-well microplate. The total number of wells that were used including 25% excess were determined and the volume of the coupled beads, detection antibodies and Streptavidin-PE needed were calculated accordingly. All the solutions, buffers and controls used during this assay was obtained from the Bio-Plex Phoshoprotein detection reagent kit *(#171-304004, BioRad Laboratories).* For step by step phosphoprotein detection assay

procedure refer to *Appendix E.7.* Data was acquired using the Luminex 100 *(version 4.1)* system and presented as arbitrary units based on fluorescent intensity.

4.3.6. Genotyping : IL-6 single nucleotide polymorphism

The Interleukin-6 locus was amplified using the following primers: 5'-TTGTCAAGACATGCCAAAGTG-3' (IL-6 sense) and 5'TCAGACATCTCAGTCCTATA-3' (IL-6 antisense)(Yamin *et al.* 2008). Polymerase chain reactions (PCR) conditions were as follows: 1x KAPA ReadyMix (*Kapa Biotech* / *Lasec*), 4pmol of each primer and 20ng DNA template and a final volume of 20μl. The PCR was performed in a Verity (*Applied Biosystems*) with the following cycling conditions: 95°C for 5 minutes followed by 35 cycles of 95°C for 30 seconds, 50°C for 30 seconds and 72°C for 30 seconds and a final extension of 72°C for 10 minutes.

Post-PCR purification was done using the NucleoFast Purification System (*Separations*). Sequencing was performed with BigDye Terminator V1.3 (*Applied Biosystems*) followed by electrophoresis on the 3730xl DNA Analyser (*Applied Biosystems*). Sequences were analyzed and trimmed using Sequencing Analysis V5.3.1 (*Applied Biosystems*). Alignments were done using the ClustalW module of BioEdit version 7.0.4.1 (*Hall, 1999*) with the downloaded SNP-ID as reference.

4.3.7 Histology : Myosin ATPase assay

The myosin ATPase assay is based on the histochemical assessment of the ATPase enzyme present on the myosin heads. Because myosin ATPase activity is related to the velocity of muscle contraction, measures of the ATPase activity can be interpreted in terms of contraction speed and therefore distinguishes between fast and slow twitch fibres. This histochemical stain is based on the different sensitivities to pH between the different myosin ATPases.

The baseline muscle biopsy samples that had been frozen in isopentane (pre-cooled in liquid N_2) were cut into 5ųm transverse sections using a *(Leica CM1100)* cryostat at temperatures between -22°C and -18°C. These sections were stained at pH 4.3 to clearly distinguish

between type I and type II fibres *(refer to Fig. 4.6, below).* For the step by step staining protocol refer to Appendix *E.9*

Slides were viewed under a light microscope *(Eclipse E400, Nikon)* and pictures were taken at x20 magnification with an integrated digital camera *(Nikon, DMX1200)* using Nikon ACT-1 software. The baseline biopsy of each participant underwent the myosin ATPase assay with an average of 494 ±286 (mean ±SD) fibres counted for each individual.

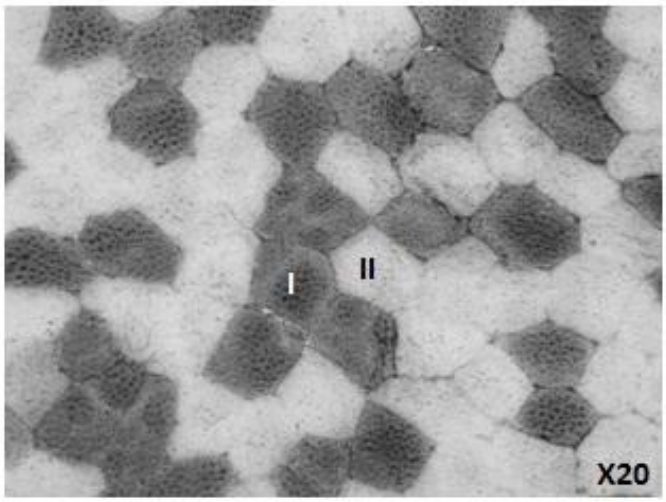

Fig 4.6 Myosin ATPase pH 4.3. Dark fibres represent type I fibres, light fibres represent type II fibres. **Abbr :** I – type I fibre, II – type II fibre.

4.3.8 Immunohistochemistry (IHC)

Cryostat sections were fixed in a 2% paraformaldehyde solution for 8 min followed by 15 minutes in 0.25% Triton X-100 *(MERCK, #1014224)* before it underwent Pax7 *(mouse monoclonal, hybridoma bank IOWA)* and Laminin *(rabbit polyclonal, DAKO Z0097,#00037479)* double staining. *(refer to Appendix E.10).*

Fluorescent microscopy was performed on the Olympus IX81 inverted fluorescent microscope and images taken at x40 magnification *(Olympus LUCPlanFLN 40x/0.60 Ph2 ∞/0-2 FN22)* using F-view-II cooled CCD camera *(Soft Imaging systems)*. The following

criteria (Thornell *et al.* 2009) were used for identifying satellite cells: stained by anti-pax7 (green), contain a nucleus stained with Hoescht (blue) and must be located in a sublaminar position confirmed by laminin (red). Co-localization of nuclei (4'6-diamidino-2phenylindale, DAPI) (blue) and pax7 (FITC) (green) were determined using ImageJ software (*Image J 1.43, Wayne Rasband, National Institute of Health, USA, rsb.info.nih@gov/ij*). The number of pax7^{+} satellite cells per fiber were determined with an average of 89 ±42 fibres counted for each individual.

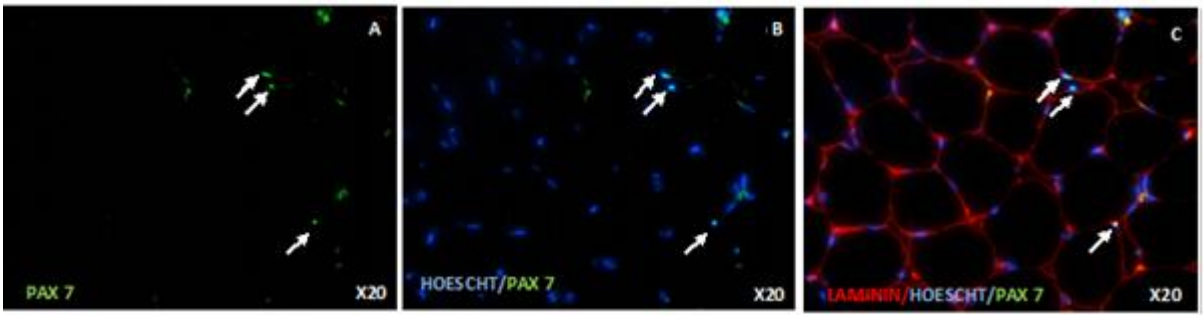

Fig. 4.7 Satellite cell count. Panel A – Indicating satellite cells stained by anti-Pax7 (FITC). **Panel B –** Indicating satellite cells stained by anti-Pax7 (FITC) and nuclei stained by hoescht (DAPI). **Panel C –** Indicating the basal lamina stained by anti-Laminin (TexasRed), satellite cells stained by anti-Pax7 (FITC) and nuclei stained by hoescht (DAPI). **Arrow** indicate Pax7^{+} satellite cells.

4.3.9 Transmission Electron Microscopy (TEM)

The muscle biopsy samples that were placed in a 3-4% paraformaldehyde solution containing 2.5% sucrose were processed and blocked for ultrastructural analysis. Transmission electron microscopy were performed in collaboration with the National Health Laboratory services (*Tygerberg Hospital, Cape Town, Western Cape*).

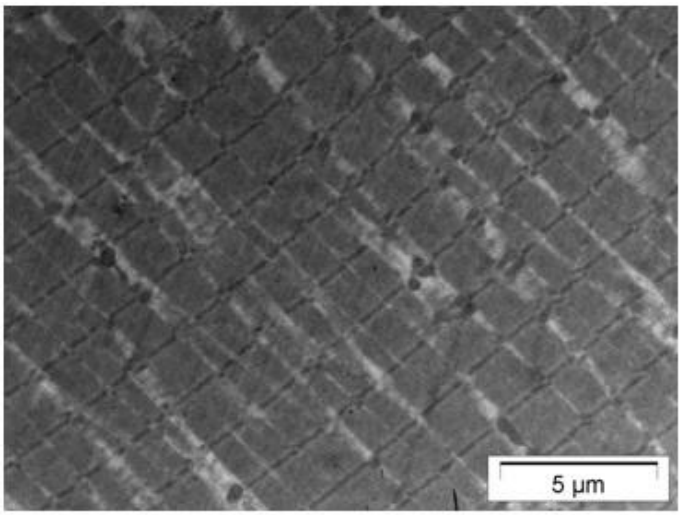

Fig. 4.8 Transmission electron micrograph. Normal sarcomere structure in a baseline biopsy of the *vastus lateralis* muscle.

4.4 Statistical analysis

All other data and statistical analysis were performed in collaboration with the Centre for Statistical Consultation, Stellenbosch University using software *(STATISTICA 10 and InStat, StatSoft)* . Values are presented as mean ± Standard Error (SE) .

Box-and-Whisker plots established the presence of any outliers more than two standard deviations from the mean. These outliers were excluded from analysis and if a specific participant showed outlier values at baseline, all time points from that particular participant were excluded from analysis for that specific variable. To determine whether a group effect existed at baseline a Kruskal-Wallis non-parametric test with Dunn's multiple comparison or Mann-Whitney nonparametric unpaired test with two tailed p-value was performed. Repeated measures ANOVA with Fisher's LSD post hoc test was used to determine significant effects of time, group or group x time. To determine effect over time in one specific subgroup an one-Way ANOVA with Fisher's post hoc test were performed. Relationships between variables were determined using Spearman's non-parametric ranked correlation analysis. The level of significance was accepted at $p<0.05$.

CHAPTER 5: HIGH INTENSITY INTERMITTENT DOWNHILL RUNNING: A MODEL FOR TESTING SENSITIVITY TO INFLAMMATION AND REGENERATIVE CAPACITY OF SKELETAL MUSCLE.

5.1 Introduction

Acute high intensity exercise leads to leukocytosis (Connolly *et al.* 2004, Nielsen *et al*. 2003, Paulsen *et al*. 2005) and significant increases in circulating pro- and anti-inflammatory cytokines (Smith *et al.* 2000). The magnitude of the cytokine response to acute exercise associated with glycogen depletion and/or skeletal muscle micro-damage is reasonably well defined and plays a role in modulating post exercise changes in immune function. In human exercise studies, despite standardization, large individual variability is a major concern. This is especially the case when investigating the relationship between inflammatory cytokines, skeletal muscle damage and regeneration.

Paulsen *et al.* (2010) demonstrated that large increases in creatine kinase (CK) *(high responders, >10 000 u.L^{-1})* are associated with greater intramuscular accumulation of radio-labelled (*99m Technetium*) leukocytes in comparison with low responders following 300 maximal eccentric contractions. The authors further observed additional CK appearance in circulation during the recovery phase *(day 4)* in the high responders. In accordance, van de Vyver *et al.* (2012) recently observed a similar biphasic response following intermittent downhill running and ruled out the biopsy procedure as possible cause for the additional leakage of muscle proteins *(CK, myoglobin (Mb), lactate dehydrogenase (LDH))* into circulation. The authors further indicated that the initial increase in markers of muscle damage coincided with elevated levels of pro-inflammatory interleukin-6 (IL-6) and tumour necrosis factor α (TNFα) suggesting a link between inflammation and muscle damage (van de Vyver *et al.* 2012).

We therefore hypothesize that two or more divergent response patterns for CK release after unaccustomed high intensity exercise might explain the variability observed in circulating markers of muscle damage and that these response patterns, are at least in part, influenced by the systemic cytokine response. Ostrowski *et al.* (1998) identified high *(>25 p.mL^{-1})* and low *(< 25 p.mL^{-1})* IL-6 responders following 2.5 hours of treadmill running *(75% VO_2max)*, but were unable to demonstrate an association between peak CK activity and plasma IL-6

concentrations. This may have been because the running protocol did not include any gradients.

More recently, Reihmane *et al.* (2012) demonstrated an association between the increase in serum IL-6 from pre to immediately post exercise *(incremental cycling to exhaustion)* and increases in both myeloperoxidase (MPO) and matrix metalloprotease-9 (MMP-9) *(both indicative of neutrophil degranulation)*. Excessive release of MPO and especially MMP-9 from neutrophils may be responsible for causing additional tissue damage, since MMP-9 is responsible for degradation of extracellular matrix components such as basement membrane collagen (Van den Steen *et al.* 2002). The pro-inflammatory cytokine response *(especially IL-6)* is thus likely to play a role in compromising sarcolemmal integrity. Although chronic inflammation is easily assessed using routine chemistry, it is not clear how to determine a propensity to develop sustained inflammation in otherwise healthy individuals. Nuclear factor kappa B (NFκB) plays a role in the transcriptional control of cytokines.

An increase in nuclear NFκB binding to IL-6 promotor regions has been shown to coincide with increases in the mRNA expression of this cytokine in the *vastus lateralis* muscle of recreationally active males 2 hours following high intensity resistance exercise (Vella *et al.* 2011). In 2009, Chiang *et al.* demonstrated that downregulation of NFκB signalling had a protective effect against exercise-induced skeletal muscle damage in rats following repeated days of downhill running. The authors suggested that this protective effect was due to the modulation of inflammation-mediated damage to muscle cells. In agreement, Lapointe *et al.* (2002) demonstrated that the accumulation of $ED1^{+}$ macrophages in rat skeletal muscle following exercise-induced muscle damage (EIMD) is responsible for inducing secondary damage and that it was prevented with the suppression of inflammation. These studies used honokoil and diclofenac respectively as their anti-inflammatory treatment.

Even though the possibility exists that molecular signalling pathways involved in the inflammatory response might be responsible for delaying or inhibiting regeneration (Lapointe *et al.* 2002, Trennery *et al.* 2008), there is strong evidence to suggest that an acute inflammatory response is essential for muscle repair. Mackey *et al.* (2007) investigated the influence of non-steroidal anti-inflammatory drugs (NSAID's) on satellite cell activity. The authors demonstrated that administration *(from 4 days before up until 8 days following exercise)* of an NSAID that limits fever, pain and inflammation by reducing the level of prostaglandins produced, has a negative effect on satellite cell numbers in humans following

exercise compared to a placebo group. This group later demonstrated that 7.5 hours of NSAID infusion into the *vastus lateralis* muscle before, during and following eccentric exercise was sufficient to suppress the exercise-induced increase in satellite cell number 8 days following exercise (Mikkelsen *et al.* 2009). The systemic cytokine response to exercise might play an essential role in initiating muscle repair. The IL-6 super-family is known to affect satellite cell proliferation (Hawke *et al.* 2001), but is unlikely to be the only mechanism. Interleukin-6 is also known to activate signal transducer and activator of transcription-3 (STAT3) signalling (McKay *et al.* 2009, Toth *et al.* 2011). We therefore aimed to evaluate inflammatory and regenerative responses following downhill running.

In addition to their role in leukocytosis, inflammatory mediators such as granulocyte colony stimulating factor (G-CSF) have also been implicated in the mobilization of bone marrow derived stem cell populations. The ability of these cells to engraft into the satellite cell niche (Blaveri *et al.* 1999, Fukada *et al.* 2002) has been confirmed in animal models where bone marrow derived stem cells contributed to skeletal muscle regeneration following severe injury (LaBarge *et al.* 2002, Palermo *et al*. 2005).

Although exercise protocols do not induce severe injury, LaBarge *et al.* (2002) demonstrated that following irradiation, transplanted GFP-labelled bone marrow derived stem cells enter the satellite cell niche and contribute to skeletal muscle regeneration after muscle damaging exercise. The same authors (Palermo *et al.* 2005) later used parabiotically joined wild type mice with genetically labelled bone marrow and indicated that stem cells originating in the bone marrow were evident in myofibres following one month of forced exercise *(extensor digitorum longus (EDL) overload)*. It was therefore concluded that bone marrow derived stem cells have the ability to contribute to myofibres in response to physiological stressors even without the presence of severe injury (Palermo *et al.* 2005).

Lethal irradiation and bone marrow transplantation have however formed a key role in the animal experimental models and definitive conclusion cannot be made regarding the potential of bone marrow derived stem cells to contribute to skeletal muscle regeneration following acute exercise in human subjects. Regular exercise training has been shown to predispose mobilization and to enhance the functional activity of circulating progenitor cells in patients with recently acquired myocardial infarction (Brehm *et al.* 2009).

A secondary objective of this study was therefore to determine whether intermittent high intensity downhill running induce mobilization of haematopoietic progenitor cells (HPC) and whether or not HPC mobilization is associated with G-CSF release in response to muscle damaging exercise.

Given the above lines of evidence we hypothesize that an early anti-inflammatory response *(interleukin-10 (IL-10))* is associated with earlier resolution of muscle damage and regeneration. Therefore the objectives of this study included the evaluation of pro-inflammatory *(serum TNFα, interleukin-1β (IL-1β), IL-6, intramuscular MPO, NFκB signalling)* and regenerative responses *(satellite cell activity, STAT3 signalling)* following downhill running.

5.2 Methodology

For in depth information on methodology and laboratory protocols refer to chapter 4 and Appendix E.

5.2.1 Subjects

Thirty healthy young men volunteered to participate in this study and were randomly subdivided into a control (n=8) and downhill run (DHR) group (n=22). All the participants were unaccustomed to any form of downhill activity and reported no exercise related injury within the previous three months. Participants were informed about the purpose and risks of the study before signing an informed consent form. The experimental protocol was approved by the Committee for Human Research at Stellenbosch University and the study was conducted according to the ethical guidelines and principles of the International Declaration of Helsinki.

5.2.2 Exercise testing

5.2.2.1 Incremental VO_2max test

Following a 5 minute (min) warm-up, all participants were asked to wear an oxygen mask linked to the metabolic system *(Oxycon Pro, Jaeger, Germany)* for breath-by-breath gas analysis. The incremental fitness test to exhaustion started at 8 $km.h^{-1}$ on a level treadmill

(*Runrace, Technogym, Italy*), where after the speed was increased by 0.5 km.h^{-1} every 30 seconds.

5.2.2.2 Downhill run

The participants in the DHR group performed a 60-min intermittent downhill run *(12 x 5 min bouts @ 15 km.h^{-1}, 10% decline)* on a motorized treadmill (*Runrace, Technogym, Italy*). Participants were allowed a 5 min standing rest between bouts and all the participants were able to complete all twelve bouts.

5.2.3 Blood sampling

Venous blood samples was collected from the antecubital vein in the supine position before (pre), immediately post and 4h post DHR on day 0 and on days 1 ,2, 3, 4 and 7 between 12:00 and 14:00 pm. Whole blood (*EDTA tubes*) samples were immediately analysed for total and differential white blood cell (WBC) count (*Cell-Dyn 3700 Diagnostech)* as well as for the expression of the CD34 fluorescein isothiocyanate *(Mouse Anti-human, #560942)*, CD38 phycoerythrin *(Mouse Anti-human, # 560981)*, CD33 allophycocyanin *(Mouse Anti-human, #551378)*, and HLA-DR peridin chlorophyll protein *(Mouse Anti-human, #347402)* antigens (*Becton Dickinson, USA*) by means of flow cytometry (*FacsCaliber BD BioSciences,SA*). Serum was analyzed using one-step sandwich assays for creatine Kinase (CK) activity *(CARDIAC Calibrator # 386371, Beckman Coulter)* and Myoglobin (Mb) concentrations *(CARDIAC Calibrator # 973243, Beckman Coulter)*. Serum cytokine concentrations (TNF-α, IL-1β, IL-6, IL-10, G-CSF) were determined using bead array (Bio-Plex Pro Human cytokine Group I assay *6-Plex, #M500002ALX, Bio-Rad Laboratories*) in combination with the luminex 100 system (*Bio-Rad Laboratories*). Serum cortisol concentrations were determined using a solid phase enzyme-linked immunosorbent assay (*EIA 1887 DRG Cortisol ELISA, # 43K041, DRG International*).

5.2.4 Needle muscle biopsy sampling

Muscle biopsies were obtained from the *vastus lateralis* muscle using a 5 mm trephine needle with assisted suction. Baseline muscle biopsies were obtained from all the participants 14 days prior to the intervention protocol. Three biopsies were obtained from four participants in

the control (n=4) and twelve participants in the DHR (n=12) group in the early recovery phase at 4h, 1 day and 2 days. Biopsy samples were divided and either preserved for cryosectioning or snap frozen in liquid N_2 and stored at -80°C.

Baseline biopsies were cut into 5µm transverse sections using a cryostat *(Leica CM1100, Leica Biosystems)* at temperatures between –22°C and -18°C. Fibre type composition were determined in these sections using myosin ATPase staining at pH 4.3 to distinguish between type I and type II fibres. Cryosections were further analysed using immunofluorescent staining with antibodies directed against laminin (*polyclonal rabbit, DAKO Z0097, #00037479,USA [1:250]*), Pax7 (*mouse monoclonal, Hybridoma bank, Iowa, USA [1:50]*) and Hoescht (*bisBenzimide H33342 trihydrochloride, 099K400, #B2261-100MG,USA [1:200]*).

Western blot analysis was used to determine the level of intramuscular MPO *(AF3174, goat polyclononal, R&D systems, WTN01 [1:250])*, myoD (*5.8A, mouse monoclonal, Santa Cruz, sc-32758 [1:500]*) and myogenin (*F5D, mouse monoclonal, Santa Cruz, sc-12731 [1:500]*), the ratio between the phosphorylated (*Y705, rabbit polyclonal, Cell Signalling, #91315 [1:500]*) and total *(Rabbit polyclonal, Cell Signalling, #9132 [1:500])* STAT3, the ratio between the phosphorylated (*sc-7977, goat polyclonal, Santa cruz, #11911 [1:1000]*) and total (*sc-1643, mouse monoclonal, Santa cruz*, #C1011 [1:1000]) IkBα. Western blot data was normalized to a percentage value with the control sample on each gel set at 100.

5.2.5 Fatigue and muscle soreness

The fatigue and muscle soreness subjects experienced were assessed *(using a report form with an annotated Borg scale (Appendix D))* before the downhill run as well as immediately, 4h and on days 1, 2, 3, 4 and 7 post intervention. The first part of the report focuses on fatigue in different muscle groups (*quadriceps, hamstrings, calves*) of the lower limb, whereas the second part of the report focuses on muscle soreness specifically in the *quadriceps* muscle group.

5.2.6 Statistical analysis

All values are presented as mean ± Standard Error (SE). Software (STATISTICA 10, StatSoft) software was used for all statistical analysis. Box-and Whisker plots were used to establish the presence of outliers defined as more than two standard deviations from the mean. These outlying data points were excluded from analysis and if a specific subject had an outlying value at baseline, all time points for that particular subject were excluded from analysis for that specific variable. To determine whether a group effect existed at baseline, a Kruskal-Wallis non-parametric test with Dunn's multiple comparison was performed. Repeated measures ANOVA with Fisher's LSD post hoc test was used to determine significant effects of time, group or group x time. For a few specific variables an effect of time in one specific subgroup was analysed using a one-way ANOVA with Fisher's post hoc test. Relationships between variables were determined using Spearman's non-parametric ranked correlation analysis. The level of significance was accepted at $p<0.05$. Note: For statistical analysis of cytokine data, control data was not used for group comparisons due to too many subjects registering non-detectable values. The abbreviations that will be used in the results section for text, tables and graphs are: *$p<0.05$, **$p<0.01$, ***$p<0.001$ indicate effect of time within each group, #$p<0.05$, ##$p<0.01$, ### $p<0.001$ indicate significant group effect at same time point.

5.3 Results

5.3.1 Subject characteristics

The subject characteristics *(mean ±SE)* (n=28) were as follow: age 20.4 ± 0.27 yr, height 1.8 ± 0.01 m, weight 76.7 ± 2.1 kg, body mass index (BMI) 23.8 ± 0.6 $kg.m^{-2}$ and maximum oxygen consumption (VO_2max) 50.7 ± 1.1 $ml.kg.min^{-1}$. The participants were randomly divided into a control (n=7) and downhill run (DHR) (n=22) group.

5.3.2 Cortisol

Serum cortisol concentrations were determined for ten participants in the downhill run group. No increase in cortisol concentration was evident immediately following the downhill run compared to baseline *(pre)* in the participants (n=8) that did not experience an exaggerated CK response *(CK>3000 u.L^{-1}, refer to section 5.3.3.1, p. 99)*. One-way ANOVA with LSD post hoc test indicated no effect of time with mean serum cortisol concentrations ranging from 83 to 172 ng.mL^{-1} throughout the study. As expected post hoc analysis indicated a significant ($p<0.05$) decline in cortisol concentration from immediately post *(09h30-10h00)* to 4h post *(13h30-14h00)* downhill running coinciding with the normal diurnal variation.

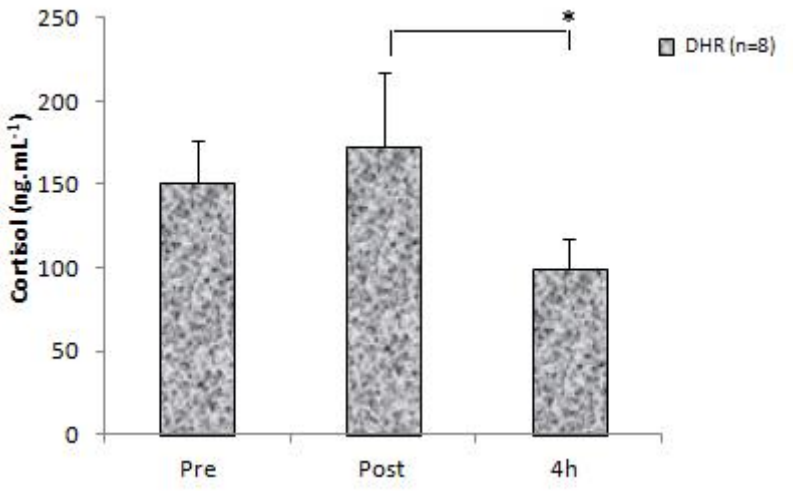

Figure 5.1 Serum cortisol *(mean ± SE)***.** Graph indicates serum cortisol concentrations for participants in the DHR group that did not experience rhabdomyolysis. **Statistical analysis:** One way ANOVA with Fisher's LSD post hoc test. *p<0.05 indicate significant decline from post to 4h. **Abbr:** DHR - downhill run, h – hour.

5.3.3 Indirect markers of muscle damage

5.3.3.1 Creatine kinase activity (CK)

Multivariate test of significance indicated no effect of time in CK activity for either the control group (n=7) or for the participants of the downhill run (n=22). This was mainly due to large individual variability (*refer to table 5.1, p. 100*) in the DHR group especially on d3 and d4. A significant group effect was however evident ($p<0.01$) with post hoc analysis

indicating significant difference between the control and DHR group from 4h to d4 following downhill running.

Table 5.1 Creatine kinase activity

	Pre	Post	4h	d1	d2	d3	d4	d7
control *(n=7)*	242 ± 75	236 ± 70	239 ± 72	247 ± 48	310 ± 62	341 ± 76	350 ± 62	242 ± 45
			##	###	#	###	###	
DHR *(n=22)*	252 ± 47	501 ± 56	1192 ± 108	1607 ± 185	1135 ± 217	1375 ± 348	1464 ± 333	923 ± 175

Footnote: Values are in units per litre (u.L-1) and presented as mean ±SE. Statistical analysis: Repeared measures ANOVA with Fisher's LSD post hoc test. # $p<0.05$, ## $p<0.01$, ### $p<0.001$ indicate significant difference between groups at the same time point. Note: Large standard errord in the DHR participants especially on d3 and d4. Abbr: d – day, DHR – downhill run, h - hour.

Upon further investigation of individual CK activity, it became clear that various response patterns were present following high intensity intermittent downhill running.

- Fourteen percent (14%) of the participants in the downhill run experienced an delayed and exaggerated peak in CK *(CK>3000 u.L^{-1})* and were grouped together as the DHR3 subgroup (n=3). A delayed and significant ($p<0.001$) peak CK activity *(4850 ± 1055 u.L^{-1})* was observed on d4 in these participants (*refer to Fig. 5.2 Panel C, p. 102*). A group effect was observed at all the time points ($p<0.001$) from 4h up to d7 between the control and DHR3 subgroup. The participants that experienced an exaggerated CK response *(DHR3 subgroup)* were excluded from all further analysis.

CK activity of the remaining eighty six percent (86%) of participants in the downhill run group peaked on d1.

- In 33% of these participants the initial peak *(1512 ± 413 u.L^{-1})* ($p<0.001$) was followed by a gradual decline returning to pre values on d7 (*refer to Fig. 5.2 Panel A, p. 102*). These participants were subsequently grouped together as the DHR1 subgroup (n=6). A significant group effect was observed at 4h ($p<0.01$) and on d1 ($p<0.001$) between the control and DHR1 subgroup.

- In 67% of the participants in which an initial peak *(1434 ± 202 u.L^{-1})* ($p<0.001$) was observed, a second significant elevation in CK activity *(1110 ± 184 u.L^{-1})* ($p<0.001$) was evident on d4. These participants were grouped together as the DHR2 subgroup (n=12). A highly significant group effect ($p<0.001$) was observed at 4h and d1 as well as on days 3 ($p<0.05$), 4 ($p<0.01$) and 7 ($p<0.01$) between the control and DHR2 subgroup. Since the group effect was not present on d2 with both DHR2 and DHR3 groups declining significantly, this suggests that a second phase of muscle damage (*refer to Fig. 5.2 Panel B, p. 102*) occurred in the DHR2 participants.

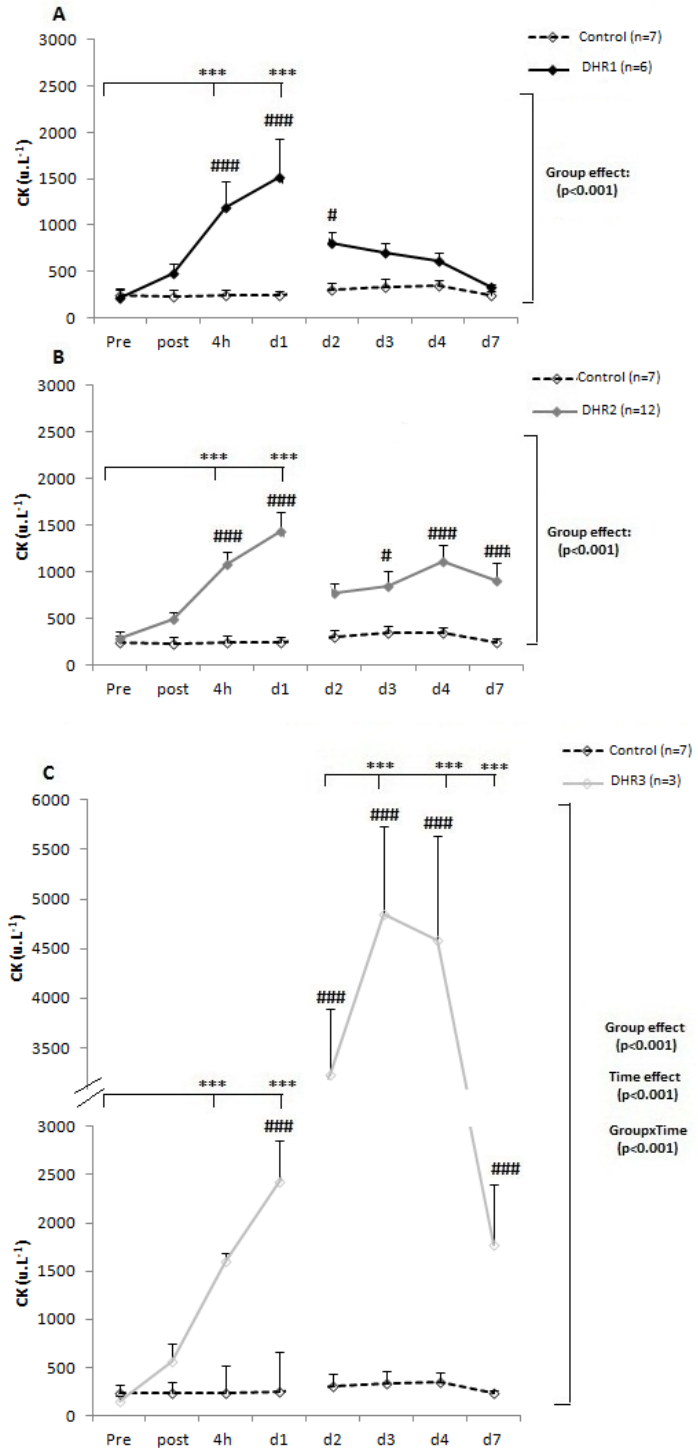

Fig 5.2 Creatine kinase *(mean ± SE)*. **Panel A** – DHR1 subgroup (n=6), **Panel B** – DHR2 subgroup (n=12), **Panel C** - DHR3 subgroup (n=3). **Statistical analysis:** Repeated measures ANOVA with LSD post hoc test. *p<0.05, **p<0.01, ***p<0.001 indicate significant difference from pre in each DHR subgroup. #p<0.05, ##p<0.01, ###p<0.001 indicate significant difference between the control and DHR subgroup at the same time point. Note: All four groups were simultaneously analysed and compared although results are presented in separate graphs. **Abbr:** CK – creatine kinase, d - day, DHR- downhill run, h – hour.

5.3.3.2 Myoglobin concentration (Mb)

Myoglobin concentrations confirmed the specific response patterns observed in CK activity. Multivariate test of significance indicated a significant effect of time, group and group x time ($p<0.001$) from pre to d1 post downhill running. Peak Mb concentrations of 357 ± 98 $ng.mL^{-1}$ and 551 ± 104 $ng.mL^{-1}$ observed in the DHR1 (n=5) and DHR2 (n=5) subgroups respectively, were followed by a sharp decline with both DHR1 and DHR2 subgroups returning to non-significant concentrations on d1. In the DHR2 subgroup there was however a second significant ($p<0.05$) elevation observed on d3 and d4, confirming that a second phase of muscle damage occurred in these participants (*refer to Fig. 5.3 Panel B, below*). Post hoc analysis indicated a significant difference between the DHR1 and DHR2 subgroups at 4h ($p<0.05$), d3 ($p<0.001$), d4 ($p<0.001$) and d7 ($p<0.05$). Note: Myoglobin concentrations were not analysed for all participants due to financial limitations.

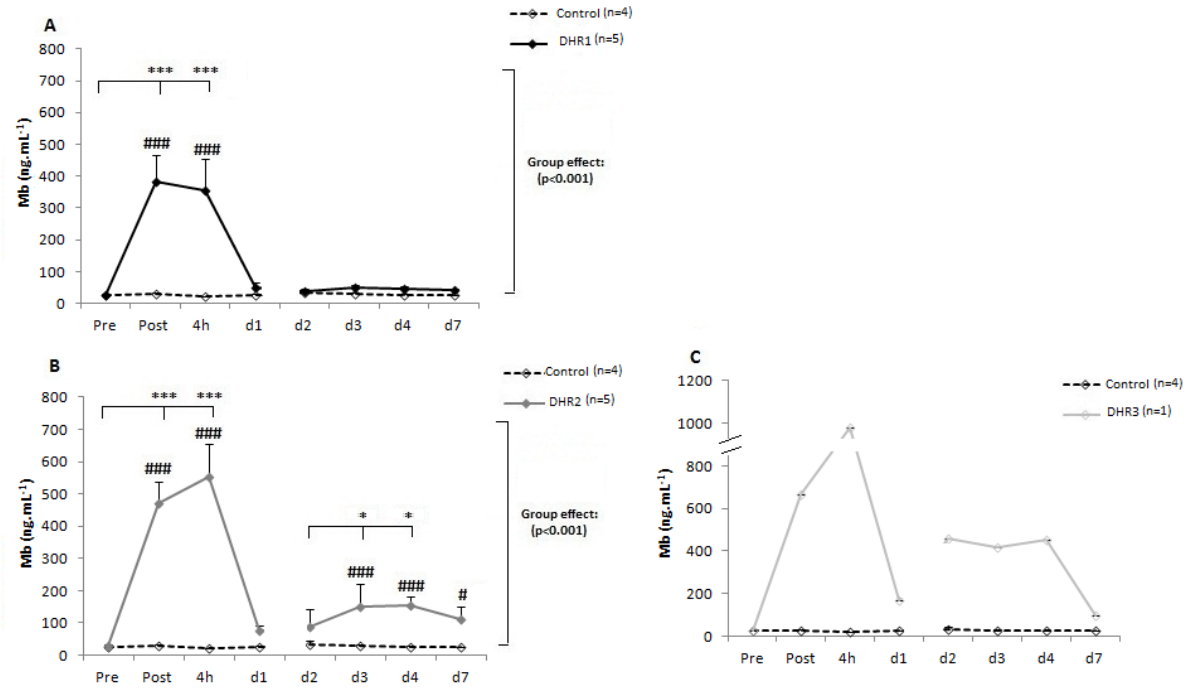

Fig 5.3 Myoglobin *(mean ± SE)*. **Panel A** – DHR1 subgroup (n=5), **Panel B** – DHR2 subgroup (n=5) **Panel C** – DHR3 subgroup (n=1). **Statistical analysis**: Repeated measures ANOVA with Fisher's LSD post hoc test. Comparing control, DHR1 and DHR2 together. Graphs are presented separately for convenience with *p<0.05, **p<0.01, ***p<0.001 indicate significant difference from pre in each DHR subgroup. #p<0.05, ##p<0.01, ###p<0.001 indicate significant difference between the control and DHR subgroup at the same time point. Note: All of the groups were simultaneously analysed and compared although results are presented in separate graphs. Control error bars are not easily distinguishable from the mean. **Abbr:** Mb – myoglobin, d- day, DHR – downhill run, h - hour.

Spearman's ranked correlation analysis indicated that myoglobin concentration at 4h was related to the second elevation in both Mb (r=0.7333, p=0.0311, n=9) on d3 and CK (r=0.800, p=0.013, n=9) on d4.

Kruskal-Wallis non-parametric with Dunn's multiple comparison test confirmed that there was no difference in age *(Control : 19.9 ± 1.6 yr, DHR1: 20.3 ± 0.8 yr, DHR2: 20.9 ± 1.8 yr, DHR3: 20.0 ± 0.0)* (p=0.541) , body mass index *(Control: 23.8 ± 4.6 kg.m^{-2}, DHR1: 23.3 ± 2.4 kg.m^{-2}, DHR2: 23.8 ± 2.4 kg.m^{-2} , DHR3: 26.4 ± 5.5 kg.m^{-2})*(p=0.6340) or fitness level *(Control: 48.7 ± 5.9 ml.min.kg^{-1}, DHR1: 50.8 ± 7.6 ml.min.kg^{-1}, DHR2: 52.6 ± 5.3 ml.min.kg^{-1}, DHR3: 50.1 ± 4.6 ml.min.kg^{-1})* (p=0.6307) between the subgroups.

5.3.3.3 Fatigue

The control group (n=7) did not experience any fatigue whereas the DHR subgroups experienced significant fatigue (p<0.01) in the muscles of the lower limbs following downhill running. A significant effect of time (p<0.01) was observed in the DHR1 subgroup up to d1 following the downhill run with participants experiencing mild to moderate overall muscle fatigue. In comparison the DHR2 subgroup experienced significant overall fatigue *(significant effect of time: p<0.01)*, fatigue in the hamstrings muscle group *(significant effect of time: p<0.01)*, quadriceps muscle group *(significant effect of time: p<0.001)* and calf muscle group *(significant effect of time: p<0.05)* up to d3 following the downhill run *(refer to Fig. 5.4, p. 105)*.

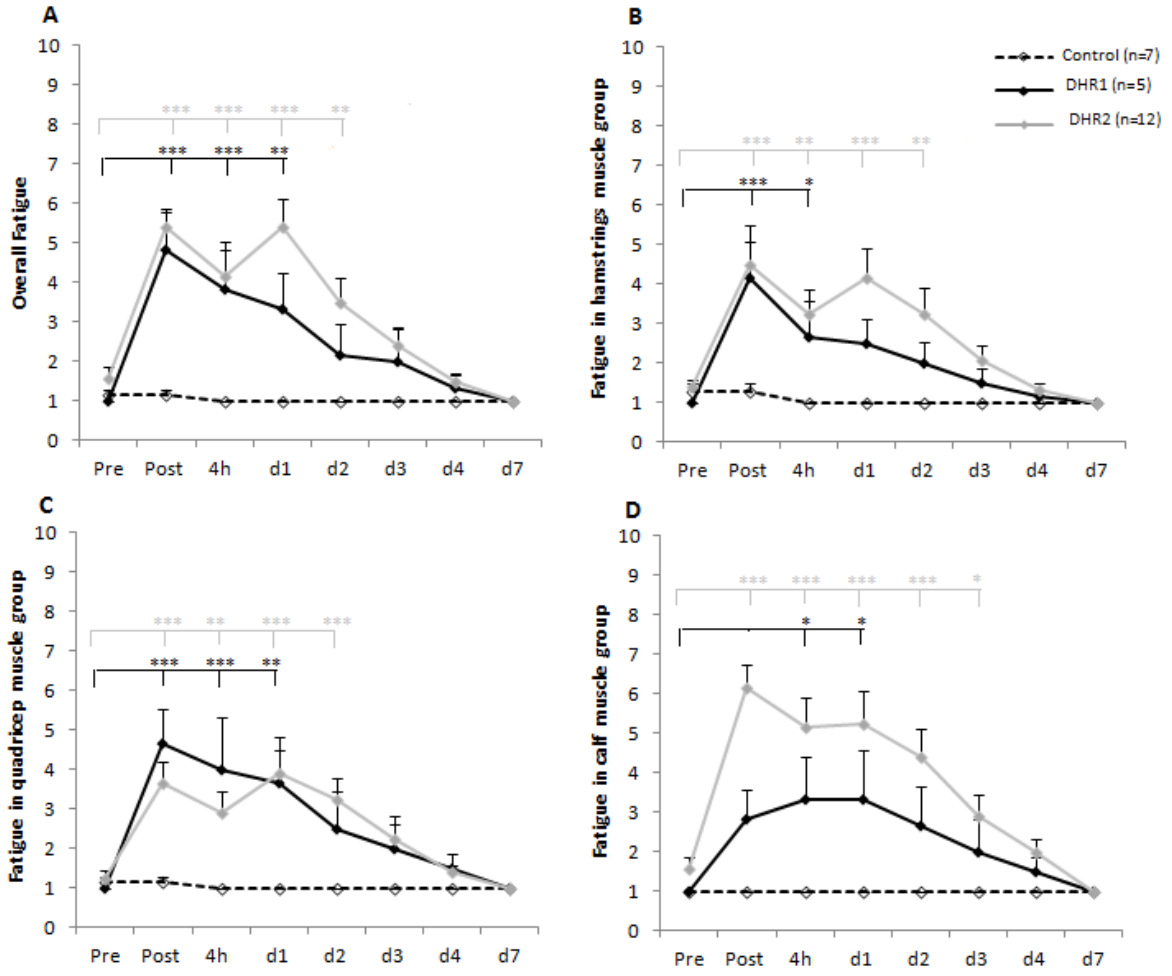

Fig 5.4 Fatigue *(mean ± SE)*. **Panel A –** Overall fatigue**. Panel B –** Fatigue in hamstrings muscle group**. Panel C –** Fatigue in quadriceps muscle group. **Panel D –** Fatigue in calf muscle group**. Statistical analysis:** Repeated measures ANOVA with Fisher's LSD post hoc test.*** $p<0.001$, ** $p < 0.01$, * $p<0.05$ indicate significant difference from pre in each DHR subgroup. No significant change over time was observed within the control group. **Fatigue rating**: 1- none, 2- very slight, 3- slight, 4- mild, 5- moderate, 6- moderate to severe, 7- severe, 8- very severe, 9- very very severe, 10- totally exhausted.

5.3.3.4 Delayed onset muscle soreness (DOMS)

The control group (n=7) did not experience any muscle soreness for the duration of the study whereas all of the DHR participants (n=21) experienced mild to severe ($p<0.001$) soreness (*refer to Fig. 5.5, p. 106*) in the *quadriceps* muscle group following the downhill run. DHR2 subgroup experienced significant soreness when applying pressure to the quadriceps muscle group on days 2 and 3. This slightly different profile was also evident in DOMS at rest.

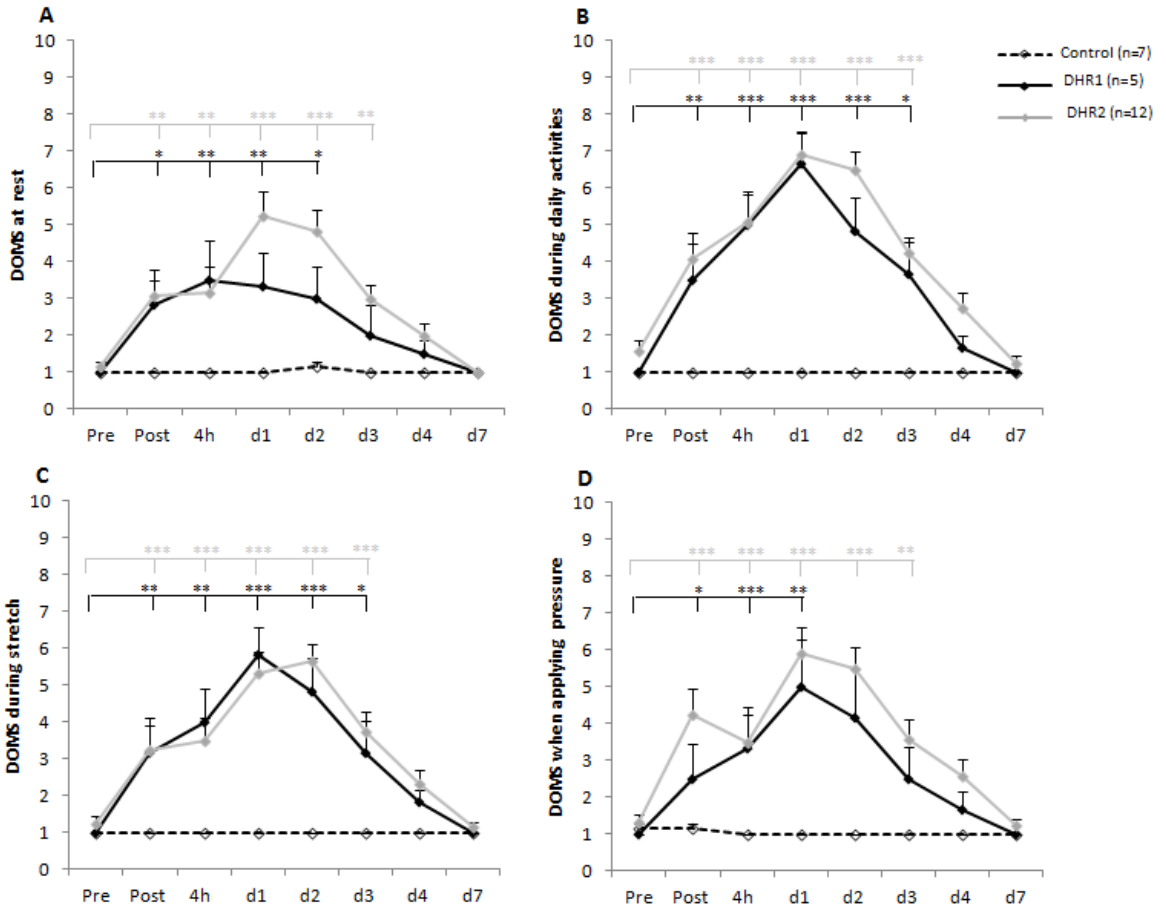

Figure 5.5 Perceived pain in *quadriceps* muscle group *(mean ± SE)***. Panel A -** DOMS at rest**. Panel B -** DOMS during daily activities**. Panel C –** DOMS during stretch**. Panel D -** DOMS when applying pressure**. Statistical analysis:** Repeated measures ANOVA with LSD post hoc test.*** p<0.001, **p < 0.01, *p<0.05 indicate significant difference from pre in each DHR subgroup. No significant change over time was observed within the control group. R**ating of perceived pain**: 1- no pain, 2- very slight, 3- slight, 4- mild, 5- moderate, 6- moderate to severe, 7- severe, 8- very severe, 9- very very severe, 10- unbearable pain.

5.3.4 Fibre type composition – vastus lateralis muscle

There was no significant difference (p=0.085) in the distribution of type I and type II fibres in the *vastus lateralis* muscle of the participants in the downhill run group. No group effect was evident between the DHR1 and DHR2 subgroups. The fibre type composition of the *vastus lateralis* muscle was however associated with evidence of the second phase of skeletal muscle damage observed on d4 indicated by the indirect serum markers of damage (*refer to section 5.3.3.1, p. 99*). CK activity on d4 were positively related to the percentage of type II fibres (*refer to Fig. 5.6 Panel C, p. 107*).

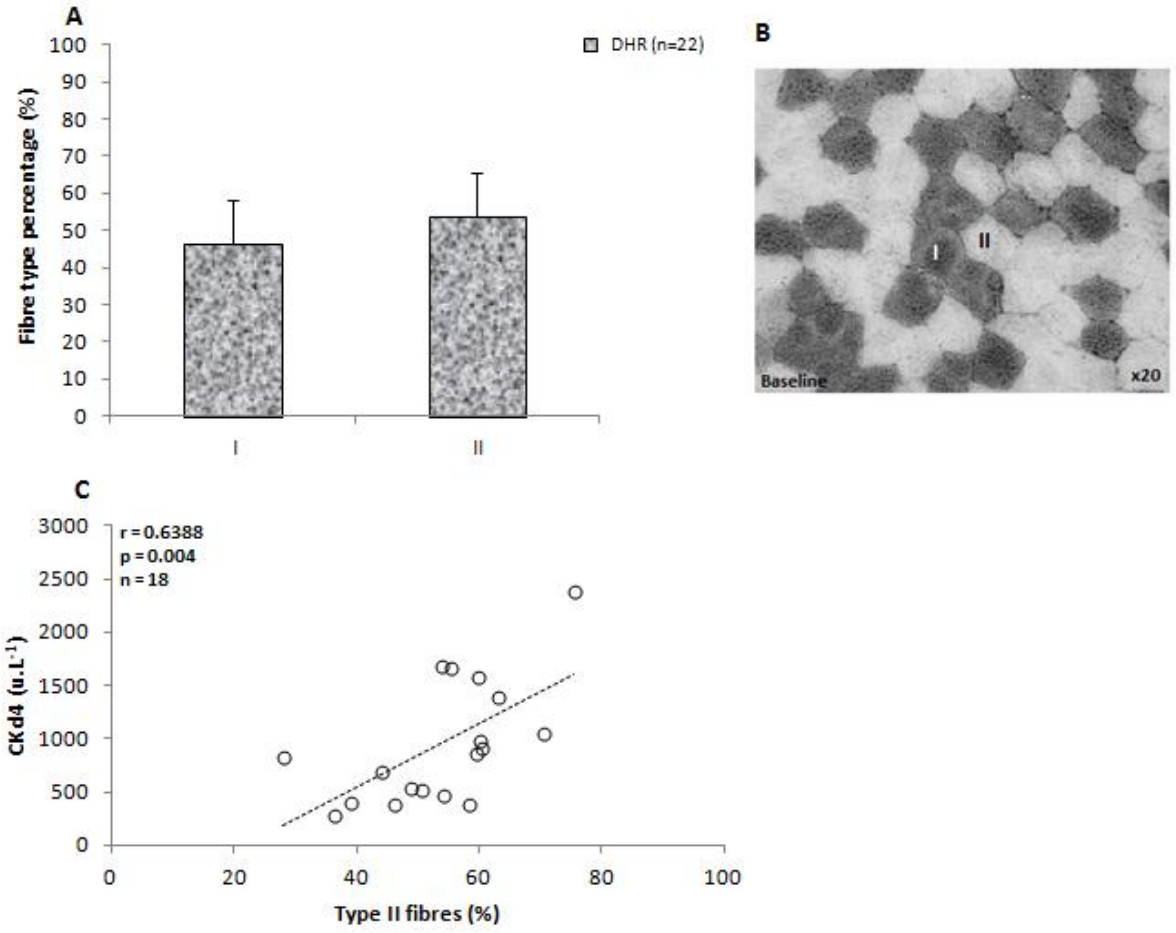

Fig 5.6 Fibre type composition of the *vastus lateralis* muscle. **Panel A –** Percentage of type I and type II fibres *(mean ± SE)* in the DHR group. **Panel B –** Myosin ATPase staining pH 4.3 viewed using light microscopy. **Panel C –** CK d4 vs type II fibres. **Statistical analysis**: Mann-Whitney nonparametric unpaired test with two tailed p value (*panel A*). Spearman's non-parametric ranked correlation analysis (*panel C*). A total of 509 ± 63 *(mean ± SE)* fibres were counted for each participant. **Abbr:** CK – creatine kinase, Mb – myoglobin, I – type I fibres, II - type II fibres.

5.3.5 Red blood cell (RBC) counts

No effect of time, group or group x time was observed for the absolute cell counts immediately before and 4h following downhill running. The haematocrit (HCT %) remained stable and therefore no corrections for plasma volume shifts were necessary *(refer to table 5.2, p. 108)*.

Table 5.2 Cell counts

	Control (n=4)		DHR1 (n=1)		DHR2 (n=7)	
	Pre	**4h**	**Pre**	**4h**	**Pre**	**4h**
RBC *(x10^{12}.L*	4.8 ± 0.3	4.6 ± 0.5	4.8 ± *n.a*	4.9 ± *n.a*	4.9 ± 0.2	4.8 ± 0.3
HGB *(g.dL^{-1})*	13.7 ± 0.7	13.4 ± 1.4	13.8 ± *n.a*	13.8 ± *n.a*	14.0 ± 1.0	14.3 ± 1.7
HCT *(%)*	41.8 ± 1.5	40.1 ± 3.4	41.7 ± *n.a*	42.6 ± *n.a*	43.0 ± 2.2	41.7 ± 3.8
MCV *(fL)*	87.5 ± 2.3	87.1 ± 2.5	86.7 ± *n.a*	86.3 ± *n.a*	87.2 ± 1.6	87.1 ± 2.7
MCH *(pg)*	28.7 ± 0.1	29.1 ± 0.1	28.7 ± *n.a*	28.0 ± *n.a*	28.4 ± 1.2	29.8 ± 1.9
MCHC *(g.dL^{-1}*	32.8 ± 0.8	33.4 ± 0.9	33.0 ± *n.a*	32.4 ± *n.a*	32.6 ± 1.4	34.2 ± 2.4
RDW *(%)*	14.7 ± 1.0	15.6 ± 1.6	15.7 ± *n.a*	14.8 ± *n.a*	14.7 ± 0.9	14.5 ± 1.3
PLT *(10^{9}.L^{-1})*	261 ± 120	250 ± 92	190 ± *n.a*	217 ± *n.a*	320 ± 81	325 ± 85
MPV *(fL)*	8.4 ± 0.9	8.8 ± 1.2	8.5 ± *n.a*	7.8 ± *n.a*	7.7 ± 0.6	7.9 ± 0.8

Footnote: Values are presented as mean ± SE. **Abbr:** n.a - not applicable, RBC – red blood cell, HGB - haemoglobin, HCT - haematocrit, MCV - corpuscular volume, MHC - corpuscular haemoglobin, MCHC - mean corpuscular haemoglobin concentration, RDW - red cell distribution width, PLT - platelets, MPV - mean platelet volume, h - hour, DHR - downhill run.

5.3.6 White blood cell (WBC) differential count

The total WBC as well as differential WBC count *(mean ± SE)* in the control group (n=7) remained within the normal clinical reference ranges *(total WBC 4.0-11.0 x10^{9} cells.L^{-1}; neutrophil 1.0-4.0 x10^{9} cells.L^{-1}; lymphocytes 1.0-4.0 x10^{9} cells.L^{-1}; monocyte 0.0-0.8 x10^{9} cells.L^{-1}; eosinophil 0.0- 0.4 x10^{9} cells.L^{-1}; basophil 0.0-0.1 x10^{9} cells.L^{-1})* throughout the study. Immediately before downhill running a normal distribution of white blood cell subpopulations *(lymphocytes, monocytes, granulocytes)* was observed using flow cytometry in the DHR participants (*refer to Fig 5.7 panel A, p. 109*).

Surprisingly, similar response patterns were observed in the two DHR subgroups, with no significant difference between these subgroups at any time point. WBC *(12.3 ± 1.9 and 12.6 ± 0.6 x10^{9} cells.L^{-1})*, neutrophil *(9 ± 1.5 and 9.2 ± 0.6 x10^{9} cells.L^{-1})* and monocyte *(1 ± 0.1 and 1 ± 0.1 x10^{9} cells.L^{-1})* count peaked at 4h post downhill running *(DHR1 and DHR2 respectively)*. This peak observed at 4h was followed by a sharp decline with a return to baseline values on d1. All the parameters remained within normal reference ranges from d2 until d7 (*refer to Fig. 5.8, p. 110*). The increase observed in total WBC count was mainly due to the increase in neutrophil count at the same time point with a highly significant correlation

(r=0.9472, p<0.001, n=17) between these two variables observed at 4h. Cell counting indicated neutrophil bands with a shift to the left. Two distinct populations of granulocytes *(flow cytometry)* were evident in 40% of the participants in the DHR2 subgroup at 4h post downhill running (*refer to Fig. 5.7 panel B, p. 109*), suggesting that a more granular population of granulocytes were present in circulation at that time point. A significant negative correlation was also observed between neutrophil count at 4h and the percentage of $CD34^+$ cells in the granulocyte region (*refer to Fig. 5.7 panel C, p. 109*).

No effect of time, group or group x time was observed in lymphocyte, eosinophil or basophil count throughout the study (*refer to Fig. 5.8 panel C, E and F, p. 110*).

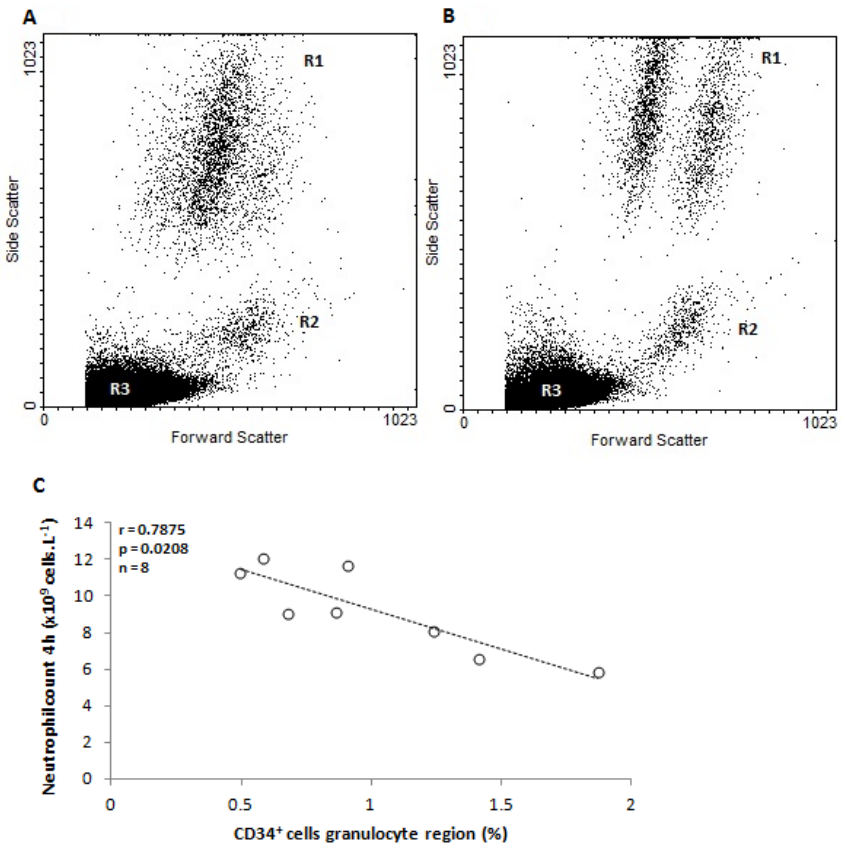

Fig 5.7 WBC subpopulations. **Panel A** – Immediately before downhill running, **Panel B** – 4 hours post downhill running. **Panel C** – Neutrophil count vs $CD34^+$ cells in granulocyte region. **Statistical analysis:** Spearman's ranked correlation analysis. **Abbr: R1**- granulocyte region**, R2 -** monocyte region, **R3** – lymphocyte region.

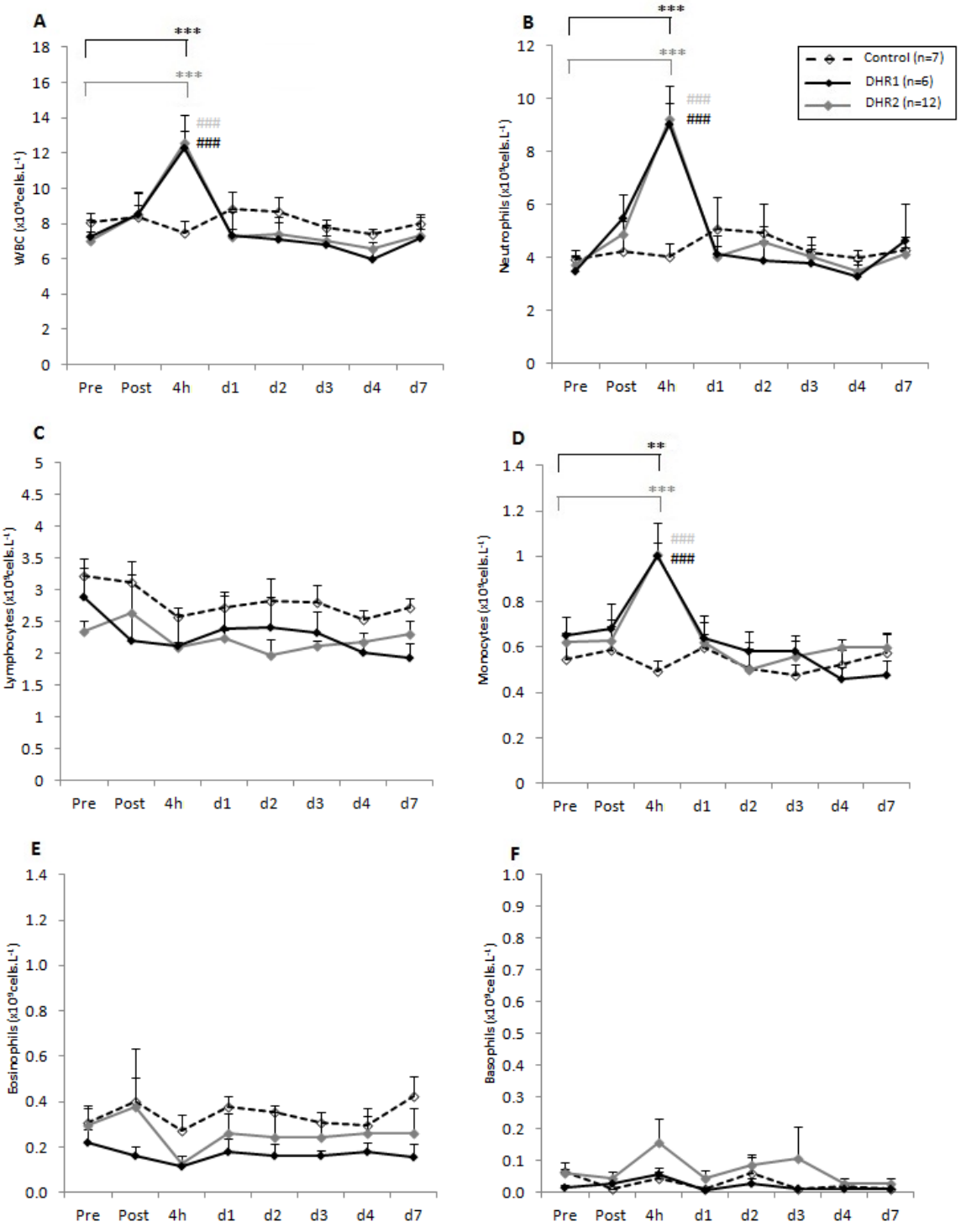

Fig 5.8 Differential white blood cell count *(mean ± SE)*. **Panel A** – WBC count, **Panel B** – Neutrophil count, **Panel C** - Lymphocyte count, **Panel D** - Monocyte count, **Panel E** – Eosinophil count**, Panel F** – Basophil count. **Statistical analysis:** Repeated measures ANOVA with LSD post hoc test. *p<0.05, **p<0.01, ***p<0.001 indicate significant difference from pre in each DHR subgroup. #p<0.05, ##p<0.01, ###p<0.001 indicate significant difference between the control and DHR subgroup at the same time point. **Abbr:** WBC – white blood cell, h – hour, DHR- downhill run.

5.3.7 Haematopoietic progenitor cells: CD34$^+$

In the DHR participants there was a significant decrease ($p<0.01$) in CD34$^+$ cells evident in specifically the granulocyte region at the 4h time point. No significant effect of time was observed in either the lymphocyte or monocyte region, with only two participants showing an elevation in the percentage of CD34$^+$ cells in the monocyte region (*refer to Fig. 5.9 below*). The DHR 1 and DHR2 subgroups behaved similarly with no clear group effect evident.

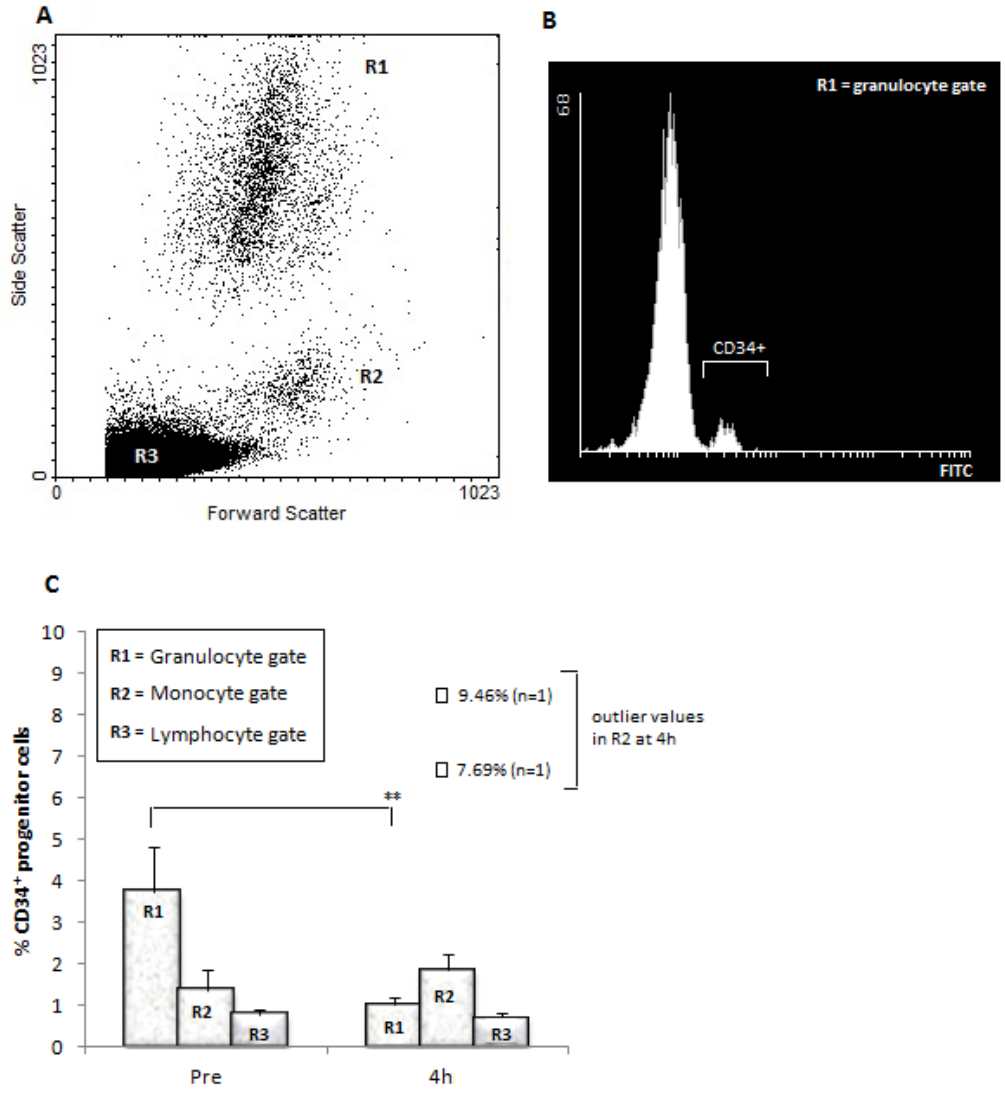

Fig. 5.9 Circulating haematopoietic progenitor cells *(mean ± SE)*. **Panel A –** WBC subpopulations. **Panel B** – Percentage of CD34$^+$ cells in each of the respective subpopulations in the DHR participants (n=8). **Statistical analysis:** One way ANOVA with Fisher's LSD post hoc test. **$p<0.01$ indicate significant difference between pre and 4h. A minimum of 5000 events were recorded and analysed. **Abbr**: d – day, DHR- downhill run.

5.3.8 Inflammatory cytokines

5.3.8.1 Tumour necrosis factor α

Serum TNFα concentrations remained below the detection limit of 4.72 $pg.mL^{-1}$ at all the time points throughout the study.

5.3.8.2 Interleukin-1β

Prior to any intervention, baseline (n=24) serum IL-1β concentration *(0.88 ± 0.63 $pg.mL^{-1}$)* was within the normal reference range. No effect of time, group or group x time was observed, with no trends in any of the DHR subgroups throughout the study.

5.3.8.3 Interleukin-6

Prior to any intervention, individual baseline (n=20) serum IL-6 concentrations were within the normal reference range *(0 - 8 $p.mL^{-1}$)*, with no effect of time observed in the control group (n=4). In the DHR1 subgroup (n=6), IL-6 concentrations peaked at 9.3 ± 4.4 $pg.mL^{-1}$ ($p<0.05$) followed by a sharp decline and returned to baseline at 4h post downhill running. A similar increase immediately post downhill running was observed in the DHR2 subgroup (n=12) but IL-6 concentrations remained elevated and peaked at 4h (10.3 ± 4.2 $pg.mL^{-1}$, $p<0.05$) before a return to baseline on d1 *(refer to Fig. 5.10, p. 113)*.

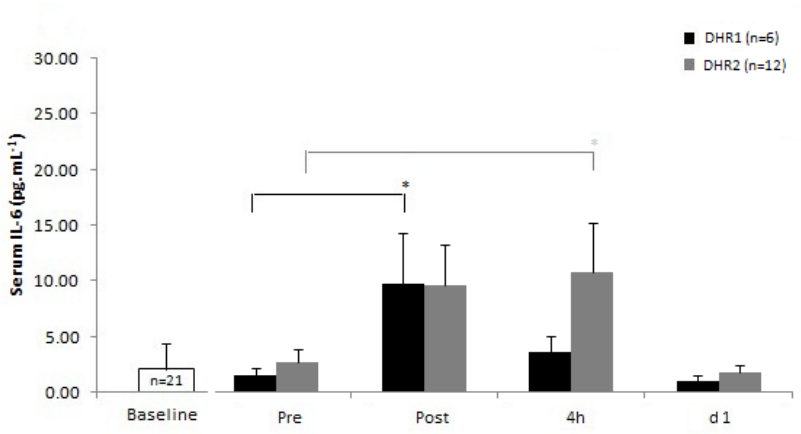

Fig 5.10 Interleukin-6 *(mean ± SE)* **Statistical analysis**: Repeated measures ANOVA with Fisher's LSD post hoc test. *p<0.05 indicate a significant change over time in each DHR subgroup respectively. **Abbr**: d – day, DHR- downhill run.

5.3.8.4 Interleukin-10

Prior to any intervention, baseline (n=22) serum IL-10 concentration was within the normal reference range *(0 - 4 pg.mL^{-1})*, with no effect of time observed in the control group (n=7). A significant (p<0.05) elevation in IL-10 concentration immediately post downhill running was evident in both the DHR1 (n=6) and DHR2 (n=12) subgroups and both groups return to pre values at 4h *(refer to Fig. 5.11, below*). Peak serum IL-10 concentrations observed immediately post downhill running were significantly correlated (r=0.9714, p<0.001, n=14) to serum IL-6 concentrations at the same time point.

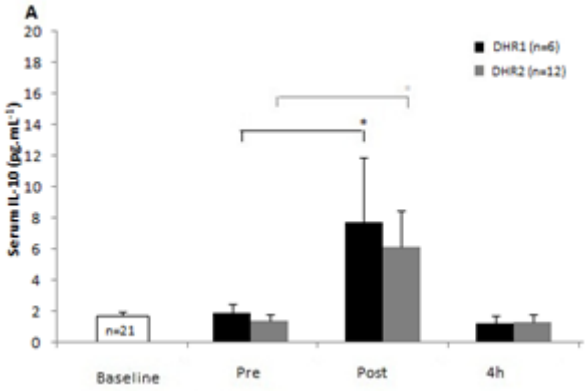

Fig 5.11 Interleukin-10 *(mean ± SE)*. **Panel A -** serum IL-10. **Statistical analysis**: Repeated measures ANOVA with LSD post hoc test. *p<0.01 indicate a significant change over time in the respective DHR subgroups. **Abbr**: d – day, DHR- downhill run.

5.3.8.5 Granulocyte colony stimulating factor

Prior to any intervention, baseline (n=24) serum G-CSF concentration were within the normal reference range *(0 - 3 pg.mL^{-1})*. Despite the DHR1 subgroup showing a slight ($p<0.05$) elevation on d3 *(6.2 ± 2.6 pg.mL^{-1})*, no effect of group or group x time was observed in either the control or any of the DHR subgroups throughout the study.

5.3.9 Intramuscular myeloperoxidase (MPO)

A significant group effect was evident between the DHR1 and DHR2 subgroups on d2 ($p<0.05$) following downhill running *(refer to Fig. 5.12 Panel A, below)*. Despite the significant group effect, no effect of time was observed in either of the DHR subgroups mainly due to large individual variability evident on d2. A highly significant relationship ($r=0.9524$, $p=0.0011$, $n=8$) was observed between intramuscular MPO levels on d1 and serum CK activity at the same time point *(refer to Fig. 5.12 Panel C, below).*

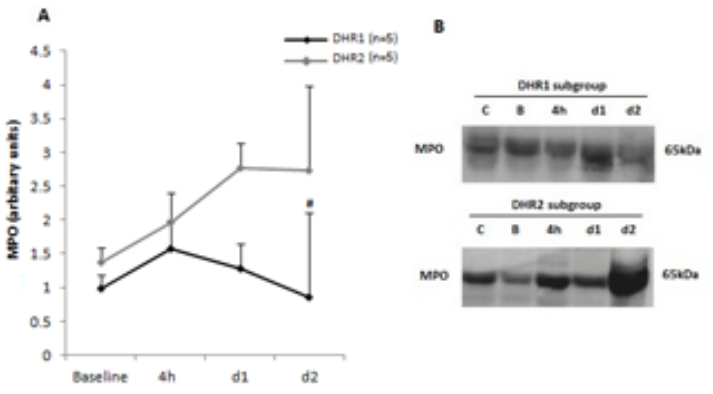

Fig 5.12 Myeloperoxidase. Panel A - Intramuscular MPO. **Panel B -** Western blot analysis. The first lane of each gel was loaded with a control sample *(uninjured muscle)*, used for the normalization of data. **Statistical analysis**: Repeated measures ANOVA with LSD post hoc test. # $p<0.05$ indicate significant difference between subgroups at same time point. **Abbr:** d- day, h- hour

5.3.10 Intramuscular NFκB Signalling

Intramuscular NFκB signalling did not differ over time for either subgroup. No effect of time, group or group x time was observed in the ratio between phosphorylated and total IκBα for either the control or any of the DHR subgroups.

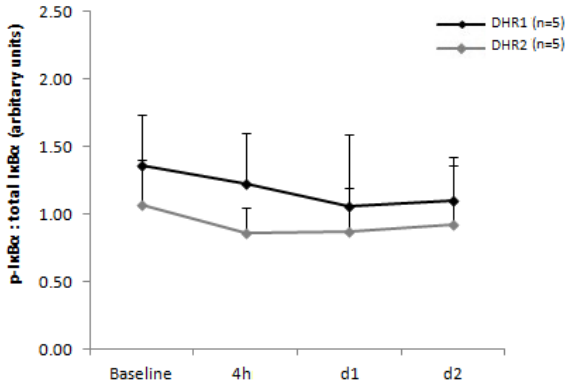

Fig 5.13 IκBα *(mean ± SE)*. **Statistical analysis**: Repeated measures ANOVA with LSD post hoc test. **Abbr**: d – day, h – hour, DHR- downhill run.

5.3.11 Intramuscular STAT3 Signalling

A significant group effect ($p<0.01$) was evident 4h following the downhill run for the intramuscular phosphorylation status of STAT3. The DHR1 (n=5) subgroup showed no change over time whereas the ratio between phosphorylated and total STAT3 was significantly ($p<0.05$) higher at 4 hours compared to d1 post downhill running in the DHR2 (n=7) subgroup (*refer to Fig. 5.14 Panel A, p. 116).* Furthermore, the intramuscular phosphorylation status of STAT3 at 4h was significantly ($r=0.850$, $p=0.003$, $n=9$) related to serum IL-6 concentrations at the same time point (*refer to Fig. 5.14 Panel C, p. 116).*

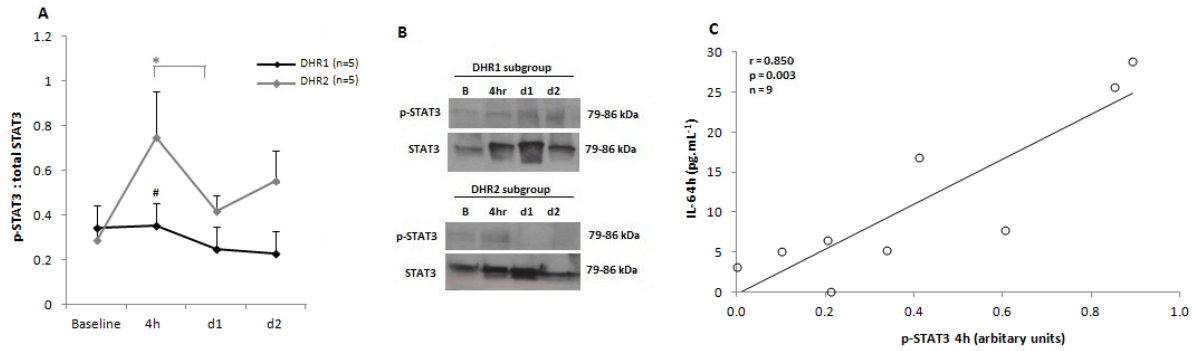

Fig 5.14 STAT3 signalling. **Panel A** - Intramuscular ratio between phospho- and total STAT3. **Panel B** - Western blot analysis. **Statistical analysis**: Repeated measures ANOVA with LSD post hoc test. * $p<0.05$ indicate significant difference between 4hr and day1 in the DHR2 subgroup. # $p<0.05$ indicate significant difference between groups at the same time point. **Panel C** – Relationship between intramuscular phosphor-STAT3 and serum IL-6 at 4 hours. **Statistical analysis:** Spearman's non-parametric correlation analysis with two tailed p value**. Abbr:** d- day, h- hour

5.3.12 Satellite cells

5.3.12.1 Pax7$^+$ satellite cells (Pax7 $^+$ SC)

No change in satellite cell count was evident in the control group throughout the study. No statistical difference in the number of Pax7$^+$ SC per fibre was observed between the DHR1 and DHR2 subgroups at any time point. Statistical analysis however indicated a significant increase in Pax7$^+$ SC per fibre on d1 in the subjects who participated in the downhill run (n=10) compared to baseline. This increase observed on d1, was followed by a sharp decline of the number of Pax7$^+$ SC which returned to baseline on d2 (*refer to Fig. 5.15 Panel B, p. 117*).

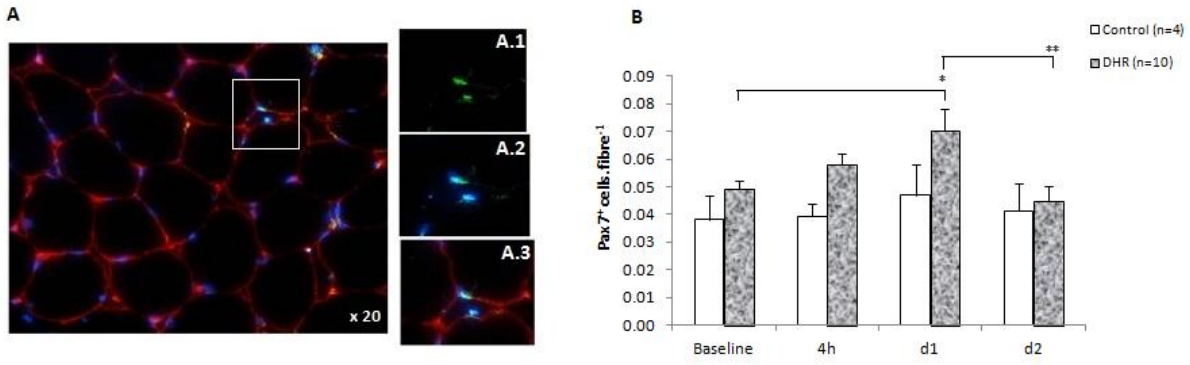

Fig 5.15 Pax 7$^+$ satellite cells per fibre. Panel A – Immunohistochemistry**. A.1** FITC – green indicating Pax 7$^+$ nuclei**. A.2** co-localization FITC and DAPI. Blue indicating all nuclei. **A.3** FITC/ DAPI and TxRed. Red indicating Laminin. **Panel B –** Number of Pax7$^+$ cells per fibre *(mean ±SE)*. Statistical analysis: Repeated measures ANOVA with Fisher's LSD post hoc test. * $p<0.05$, indicate significant change from baseline to d1 and ** $p<0.01$ indicate significant difference between d1 and d2 in the DHR group.

5.3.12.2 Myogenic regulatory factors (MRF's)

Multivariate analysis of variance indicated that no group effect existed between the DHR1 and DHR2 subgroup at any time point for intramuscular myoD and/or myogenin protein levels. Furthermore, no change over time was observed in the content of these MRF's in the participants who participated in the downhill run *(refer to table 5.3, below)*.

Table 5.3 Intramuscular content of myogenic regulatory factors

	Baseline	4h	d1	d2
myoD *(DHR n=10)*	0.85 ± 0.4	0.88 ± 0.71	1.2 ± 0.87	0.9 ± 0.61
myogenin *(DHR n=*	0.39 ± 0.27	0.43 ± 0.33	0.50 ± 0.36	0.40 ± 0.26

Footnote: Values (arbitary units) are presented for all the subjects who participated in the downhill run. **Statistical analysis:** One way ANOVA with Fisher's LSD post hoc test. **Abbr**: d - day, h - hour

5.4 Discussion

An important aspect of this study which used an intermittent downhill run protocol was the ability to identify a subset of participants who experienced, at 3 to 4 days post downhill running, a second rise in circulating markers of muscle damage (*DHR2 subgroup*), while others returned to normal levels (*DHR1 subgroup*). By subdividing the participants based on their individual CK and Mb release over time, the large standard deviations usually observed following EIMD were significantly reduced.

A similar biphasic response was previously associated with significant intramuscular leukocyte accumulation in a small number of participants who also experienced an exaggerated initial increase in CK release (*CK activity >10 000 u.L^{-1}*) (Paulsen *et al.* 2010). Higher intramuscular MPO indicative of neutrophil accumulation in the DHR2 subgroup in comparison with the DHR1 subgroup together with the MPO correlation with CK activity, support the findings of Paulsen *et al.* (2010). One difference between the two studies is that this study excluded subjects with exaggerated CK release which was indicative of a response similar to rhabdomyolysis. This resulted in an early increase in CK and Mb *(up to d1)* that was similar for all participants in the present study and the divergent response patterns only became evident from d2 to d7 following downhill running. Myoglobin concentration at 4h was related to the second elevation in both Mb *(d3)* and CK activity *(d4)*, suggesting that a higher initial response might be indicative of a subsequent, delayed secondary response. Further evidence that this was a secondary response and not merely a delay in disappearance from circulation was that two days post downhill running there was no difference between the DHR1 and DHR2 subgroups. Additional markers of damage *(CK, Mb)* were thus released into circulation in the late recovery phase. In accordance with our previous work (van de Vyver *et al.* 2012) the use of a non-exercising control group demonstrated that the biopsy procedures did not influence the appearance of CK or Mb in circulation. Compared to the previous study the subjects in this study also had a very narrow range for VO_2max indicating that this phenomenon is not related to differences in ability or fitness.

In the subset of participants *(DHR2 subgroup)* that experienced a second phase of muscle damage, it is relevant that additional IL-6 was produced following the cessation of exercise with a delayed peak observed at 4h. Interleukin-6 serves a metabolic function during exercise. Arterial femoral venous differences in IL-6 concentration during exercise *(180 min,*

two legged knee extention) (Steensberg *et al*. 2002) demonstrate that exercising limbs release IL-6 even without the presence of skeletal muscle damage (Croisier *et al*. 1999, Willoughby *et al.* 2003). Either a decrease in muscle glycogen (Gusba *et al.* 2008) content or changes in intracellular calcium levels (Keller *et al.* 2001) can signal the production of IL-6 through a number of transcription factors including p38 mitogen activated protein kinase (MAPK), Janus kinases (JNK), nuclear factor of activated T-cells (NFAT) and transcription factor AP-1 (AP1) (Febbraio *et al.* 2002, Pedersen *et al.* 2001). During prolonged exercise, glycogen depletion result in IL-6 production that in turn increases hepatic glucose output and the number of GLUT4 transporters (Banzet *et al.* 2009). This has been confirmed in studies showing that glucose ingestion during exercise decrease the exercise induced increase in IL-6 and inhibits IL-6 release form skeletal muscle (Febbraio *et al.* 2003). The immune response to exercise is also reduced with carbohydrate ingestion. Carbohydrate ingestion attenuated lymphocytosis following acute resistance exercise (Carlson *et al.* 2008) and neutrophil counts following acute cycling *(90 min @ 60%VO2max, 1.6km time trail)* in hot conditions (Peake *et al.* 2008). Nonetheless, this does not negate the pro-inflammatory function of this cytokine and it is still accepted that IL-6 has immune functions.

The production of IL-6 *(DHR2 subgroup)* after the termination of exercise occurred independently of muscle contraction. Although it coincided with a peak in circulating neutrophil and monocyte count there was a similar leukocytosis response between subgroups. At least two possible reasons can be put forward. The increased systemic cytokine response explained by a more activated status of the neutrophils or monocytes or both (Nieman *et al.* 2006). Secondly, the additional IL-6 may be muscle derived, either synthesized by myonuclei or released by resident immune cells (Croisier *et al*. 1999, Nieman *et al.* 2006, Willoughby *et al.* 2003).

Single nucleotide polymorphisms (SNP's) can influence the susceptibility of healthy individuals to exercise induced muscle damage and lead to an exaggerated CK response. Certain genotype variants of the angiotensin I converting enzyme (ACE) has been shown to be an independent determinant of peak CK activity following eccentric exercise of the elbow flexors (Yamin *et al.* 2007). Also, the muscle specific creatine kinase (CK-MM) *Ncol* polymorphism is thought to be associated with an increased risk of exaggerated CK response to exercise (Heled *et al.* 2007). A strong association between homozygosity for the IL-6 174C allele and the systemic CK response (Yamin *et al.* 2008) has also been demonstrated.

However neither of these proteins would be expected to explain the secondary inflammatory response. Individual homozygosity for the IL-6 174C allele was unrelated to the divergent CK response patterns in the present study. Data from participants experiencing a delayed CK peak on d4 that was greater than 3000 $u.L^{-1}$, were excluded from analysis since an exaggerated CK response might have been associated with underlying myosis or certain genetic factors (Capacchione *et al.* 2010, Hubal *et al.* 2010, Kenney *et al.* 2012, Landau *et al.* 2012, Sambuughin *et al.* 2009).

Previously, individual variability evident in circulating markers of muscle damage during the early recovery phase has been ascribed to gender differences, age, intensity and duration of exercise, training history of participants and/or fibre type distribution (Arnett *et al*. 2000, Epstein *et al.* 2006, Fredsted *et al.* 2008, Magal *et al.* 2010, Miles *et al*. 1994, Roth *et al.* 2000). Type II fibres are more susceptible to eccentric exercise-induced muscle damage (Macaluso *et al.* 2012), but investigations into the relationship between increases in serum CK activity and muscle fibre composition have failed to confirm an association (Magal *et al.* 2010). Possibly because the intramuscular damage is difficult to quantify. Similarly, the present study failed to demonstrate an association between the initial increase in indirect markers of muscle damage and fibre type distribution of the *vastus lateralis* muscle. A higher percentage of type II fibres did however seem to predispose participants to a second phase of muscle damage *(unrelated to the initial mechanical insult)*, since both CK activity and Mb concentrations on d4 were associated with a higher percentage of type II fibres. It is important to note though that the time course of the DHR1 and DHR2 response patterns may be specific to downhill running, since other forms of eccentric exercise could result in a delayed initial peak in CK sometimes observed as late as day 4 and day 6 (*for review see* Baird *et al.* 2012).

Consistently the literature indicates very low or even undetectable serum/plasma TNFα and IL-1β concentrations following acute exercise in healthy young men *(refer to table 5.4, p. 125*). Current data are in agreement, but also further indicates no change in intramuscular NFκB signalling in either of the DHR subgroups. A delayed peak in IL-6 concentrations *(2-8h post)* following muscle damaging exercise in comparison with increased levels of IL-6 peaking immediately post concentric exercise has also been a consistent finding *(refer to table 5.4, p. 125*). The current study is the first to show that a delayed peak is not a consistent inter-individual finding and that in those without this response, no secondary increase in CK

release is present. No change in the IL-6 response to acute exercise is evident following training despite significant reductions in muscle soreness and serum markers of muscle damage (Croisier *et al*. 1999, Willoughby *et al.* 2003) suggesting that no direct association between IL-6 and muscle damage exists. Steensberg *et al.* (2003) demonstrated that infusion of a low dose recombinant human (rh) IL-6 *(corresponding to levels obtained during strenuous exercise)* induced an anti-inflammatory environment by stimulating the synthesis of anti-inflammatory IL-10 and IL-1 receptor antagonist (IL-1ra). This might explain the absence of serum TNFα and IL-1β following acute exercise. In agreement, Ostrowski *et al.* (1998) demonstrated that in high responders peak IL-6 concentrations observed immediately post exhaustive treadmill running is followed by significant increases in plasma IL-1ra concentrations at 2 hours post exercise. In the present study increased serum IL-6 concentrations immediately post downhill running were closely associated and coincided with increases in anti-inflammatory IL-10. Interleukin-10 has a variety of anti-inflammatory mechanisms of action. In the context of tissue damage, IL-10 has been associated with inhibition of pro-inflammatory cytokine production through signal transducer and activator of transcription / suppressor of cytokine signalling (STAT3/SOCS) pathway (Murray *et al.* 2006, Schroder *et al*. 2003) and the induction of monocyte differentiation into anti-inflammatory macrophages that clear cellular debris without releasing additional pro-inflammatory mediators (Allavena *et al.* 1997, 1998). In both subgroups, anti-inflammatory IL-10 returned to pre values at 4h following downhill running despite the additional appearance of IL-6 in circulation in the DHR2 subgroup.

In the study by Steensberg *et al.* (2003), 2 hours of (rh)IL-6 infusion was furthermore associated with a significant increase in neutrophil count, suggesting that IL-6 might have an central role in exercise-induced leukocyte trafficking. This is supported by Yamada *et al.* (2002) demonstrating that plasma IL-6 *(1 hour post maximal exercise test on level treadmill)* significantly correlates with the number of circulating neutrophils *(2 hours post exercise).* Based on significant associations between markers of neutrophil degranulation *(MPO and MMP-9)* and IL-6 following maximal cycling to exhaustion, Reihmane *et al.* (2012) recently suggested that neutrophils could be the main source of inflammatory biomarkers during maximal exercise. However, following the cessation of exercise an increase in the number of monocytes that respond to *ex vivo* lipopolysaccharide (LPS) stimulation by producing IL-6 has also been demonstrated (Starkie *et al.* 2001).

Rhind *et al*. (2001) furthermore indicated that the proportion of $CD14^+$ monocytes exhibiting spontaneous intracellular expression of IL-6 increased following seven days of exhaustive exercise. Individual transcriptional changes in circulating leukocytes following acute exercise may therefore translate into divergent systemic responses to exercise and warrants further investigation. Microarray analysis of the transcriptional output of peripheral blood mononuclear cells (PBMC's) following acute exercise demonstrated that the greatest transcriptional changes occur in pathways related to the immune response and inflammation (Carlson *et al.* 2011, Connolly *et al.* 2004, Radom-Aizik *et al.* 2009). By studying the effect of eccentric exercise on NFκB signalling in PBMC's, Garcia-Lopez *et al.* (2007) indicated increased NFκB activation coinciding with increased p-IκBα and p-IKK protein levels following an acute bout of eccentric exercise. Similarly, Jimenez-Jimenez *et al.* (2008) demonstrated a significant increase in p65/p50 activity and the phosphorylation of IκBα in PBMC's following an acute unaccustomed bout of eccentric exercise. A similar increase in monocyte count was however observed in both the DHR subgroups, suggesting that if PBMC's were responsible for the production of additional IL-6, their activation status might have differed between the two DHR subgroups.

Previously, using a similar downhill run protocol, we presented qualitative evidence of immune cell attraction and invasion, as well as phagocytosis including membrane disruption (van de Vyver *et al.* 2012). To assess the possible damaging effects of neutrophils and monocytes this study investigated changes in WBC count, inflammatory cytokines and intramuscular MPO and NFκB signalling in relation to indirect markers of muscle damage. No change in NFκB signalling raises the question on whether the more prolonged elevation of IL-6 evident in the early recovery phase, had any effect on events occurring during the late recovery phase.

The downstream effects of IL-6 are dependent on its interaction with either a membrane bound (gp130) or soluble receptor (sIL-6R) (Robson-Ansley *et al.* 2010). The binding of IL-6 to the membrane bound receptor ultimately results in the nuclear translocation of activated STAT proteins which affect gene transcription of proteins related to inflammation (Trennery *et al.* 2008), proliferation (McKay *et al.* 2009, Toth *et al.* 2011) and/or angiogenesis (Cheranov *et al*. 2008). These three processes were addressed briefly in the current study by assessment of STAT3, Pax7 and CD34 respectively. The interpretation of these data are not simple, but a few points of discussion will be put forward.

In the present study the DHR2 subgroup showed significant intramuscular STAT3 signalling at 4h with the phosphorylation status of STAT3 associated with serum IL-6 at the same time point. An increased STAT3 signalling responsiveness to pro-inflammatory factors has been hypothesized to impact on mechanisms of muscle repair (Trennery *et al.* 2008) especially in aged muscle. Instead some participants *(DHR2 subgroup*) seem to have a less efficient capacity to control the post damage inflammatory response. The lack of additional IL-10 production at 4h suggests that the pro-inflammatory properties of IL-6 lead to a systemic inflammatory response in the DHR2 subgroup that ultimately resulted in a second phase of muscle damage. Serum IL-6 concentrations in the DHR2 subgroup did however return to pre values on d1 post downhill running, most likely due to negative feedback regulation (Krebs *et al.* 2001, Murray *et al.* 2006, Trennery *et al.* 2008). In some studies IL-6 induction of STAT3 signalling has been shown to be exclusive to the nuclei of satellite cells (Toth *et al.* 2011) and to correlate with increases in satellite cell proliferation in response to acute muscle lengthening contractions (McKay *et al.* 2009). Despite a significant group effect at 4h with regards to the phosphorylation status of intramuscular STAT3, both the DHR subgroups showed evidence of satellite cell activation within 24 hours following downhill running. Satellite cell activation was however not sustained, with no evidence of regulation by MRFs. This suggests that there was no difference in the regenerative response between the two DHR subgroups.

A time-dependant release of progenitor cells during and following 4 hours of cycling (Mobius Winkler *et al.* 2009) have been demonstrated. To determine whether exercise intensity might modulate progenitor cell release, Bonsignore *et al.* (2010) measured both hematopoietic and angiogenic progenitors following a marathon and 1.5 km field test in healthy young men. Maximal exercise was associated with the release of both hematopoietic and endothelial progenitors whereas endurance exercise resulted in increased endothelial and reduced hematopoietic progenitors in circulation (Bonsignore *et al.* 2010). The same authors previously (Bonsignore *et al.* 2002) reported that amateur runners had up to 4 fold higher circulating hematopoietic progenitor cells compared to sedentary controls at rest. Based on this observation the authors suggested that bone marrow activity might be modulated by the inflammatory mediators and stressors during habitual running. Supporting this hypothesis, Spiropoulos *et al.* (2010) indicated that exercise-induced inflammation has the potential to modulate bone marrow homeostasis. However, Adams *et al.* (2008) indicated a significant

decrease in circulating progenitor cells accompanied by an acute inflammatory response in well-trained older men following a marathon race.

Similarly a decrease in $CD34^+$ cells specifically in the granulocyte region with no significant changes in G-CSF concentrations were evident 4h following downhill running in the present study. Although these studies appear to be contradictory, it is known the $CD34^+$ granulocytes can mature into neutrophils. A correlation was observed between peak circulating neutrophils and the decrease in $CD34^+$ cells in the granulocyte region at the same time point. This may explain the immature population of granulocytes evident following the downhill run. An hypothesis can be put forward to explain the current data. Despite only 4-5% $CD34^+$ granulocytes, the granulocytes quickly lost the expression of CD34 with the resultant neutrophils still not mature. It is possible that these neutrophils may remain in circulation, mature over time and contribute to the secondary phage of damage. The preliminary data *(d0-d2)* discussed here for the three different lines of investigation are sufficient to embark on further studies to investigate not only the functional activity of mobilized leukocytes but also inter-individual variability in anti-inflammatory cytokine responses.

Table 5.4 Cytokine response to exercise

First Auth, date	Intervention protocol	TNFα	IL-1β	IL-6	IL-10
Concentric exercise		***peak time***	***peak time***	***peak time***	***peak time***
Bruunsgaard 1997	Cycling 30min @65% VO_2max	n.d	n.d	no change	_
Ostrowski 1998	2.5h treadmill @75% VO_2max	no change	no change	post	_
Peake 2005	level treadmill 85% VO_2max	_	_	post	1h
Sorichter 2006	60 minlevel treadmill @85% VO_2max	_	_	post	_
Gokhale 2007	3 x 20 min running	post	_	post	_
Wang 2007	graded cycling until exhaustion	no change	reduced	no change	no change
Peake 2008	90min cycling 65% VO_2max	no change	_	post	post
Eccentric exercise					
Nosaka 1996	24 max ecc rep elbow flexord	_	_	no change	_
Bruunsgaard 1997	Ecc cycling 20min @100% VO_2max; 10min @ 150% VO_2max	n.d	n.d	2h	_
Hellsten 1997	5 bouts of one-legged ecc ex	_	_	1.5h	_
Croisier 1999	ecc isokenetic ex	_	_	24h	_
Smith 2000	ecc benc pres & leg curl 4 sets of 12 reps @ 100% RM	no change	reduced	24h	48h
MacIntyre 2001	quadriceps ecc ex 300 reps	_	_	6h	_
Steensberg 2002	knee-extensor ex 180min	no change	_	3h	_
Toft 2002	60 min lower limb ecc ex	_	_	4h	_
Willoughby 2003	7 sets of 10 repsknee-extensor @150% 1RM	_	_	6h	_
Hirose 2004	elbow flexors dumbbell 90% flexed 6 sets of 5 reps	no change	no change	3h	no change
Paulsen 2005	quadriceps ecc ex 300 reps	_	_	6h	_
Miles 2008	elbow flexors 3 sets of 15 reps@ 1RM	_	_	8h	_
Afroundeh 2010	3 sets of 10 elbow flexion	_	_	8h	_
Robson-Ansley 2010	6 sets of 10 reps knee flexion	_	_	No change	_
Downhill running					
Pyne 1997	8x5min DHR @52% VO_2max	_	no change	_	_
Peake 2005	10% DHR 45min 65% VO_2max	_	_	1h	1h
Simpson 2005	7km mountain hill race	no change	_		_
Smith 2007	13.5% DHR 60 min	no change	no change	6h	6h
Broadbent 2010	40 min DHR	_	_	24h	_

Footnote: The table include all studies that fit the following criteria: subjects are healthy young men, exercise intervention include concentric; eccentric; downhill running protocols NOT endurance exercise, no supplementation or other form of intervention, cytokines analyzed in serum or plasma. **Abbr:** DHR - downhill run, ecc - eccentric, ex - exercise, h - hours, IL - interleukin, min - minutes, n.d - not detectable, RM - repitition maximum, TNF - tumour necrosis factor.

5.5 Conclusion

This study clearly demonstrates that young healthy individuals, exposed to muscle micro-damage from unaccustomed eccentric exercise can be divided into those with a adequate and those with a less efficient capacity to control post-damage inflammatory processes. This finding is important because it allows for future experimentation on the integration between the nature of muscle damage and the inflammatory response without large inter-individual variability masking the true effects not reflected when interpreting the whole population. The use of a non-exercising control group demonstrated that the events observed during the recovery phase were as result of the downhill running intervention protocol and subsequent muscle micro-damage.

The present study demonstrates that the additional production of immune related interleukin-6 is associated with the downstream events involved in a more sustained inflammatory response. As consequence sarcolemmal integrity were further compromised during the late recovery phase. No direct association between IL-6 and muscle damage were however evident. The cytokine response to downhill running is complex and not easily explained. The exercise stimuli and subsequent skeletal muscle damage was sufficient to induce an immediate IL-6 and IL-10 response. The anti-inflammatory response was however not sustained. The cytokine response during the first few hours following the cessation of exercise should be investigated further, since the events occurring during the early recovery phase seem to be indicative of events to follow during the late recovery phase.

CHAPTER 6: RELATIONSHIP BETWEEN SECONDARY SKELETAL MUSCLE DAMAGE, LEUKOCYTOSIS AND THE CYTOKINE RESPONSE TO HIGH INTENSITY INTERMITTENT DOWNHILL RUNNING.

6.1 Introduction

Delayed secondary muscle damage after an initial mechanical insult from unaccustomed eccentric exercise is a consequence of oxidative stress but may also be associated with an exaggerated inflammatory response. (Cooper *et al.* 2007, Falone *et al.* 2010, Lapointe *et al.* 2002, Paulsen *et al.* 2010). Based on evidence from *in vitro* and animal model experiments it is known that activated immune cells that have infiltrated tissue are usually accompanied by varying degrees of tissue destruction (Brickson *et al*. 2003, Lapointe *et al.* 2002, Nguyen *et al.* 2003, 2005). Neutrophils generate high quantities of cytotoxic superoxide and hypochlorous acid *(myeloperoxidase (MPO) dependent reaction)*, whereas the production of nitric oxide (NO) by pro-inflammatory macrophages is also cytotoxic (Albina *et al.* 1990, Nguyen *et al.* 2003, Toumi *et al*. 2006). However these factors causing damage are also required for regeneration to proceed. The dilemma is to determine if the secondary damage is excessive or not. *In vitro* studies in human subjects are unlikely to shed light on the mechanisms underlying these phenomena without more invasive investigation and an integrative interpretation of data obtained from *in vitro* and animal models.

Mechanical loading of skeletal muscle promotes the activation and cytotoxic capacity of neutrophils. Nguyen *et al.* (2005) demonstrated that cyclic mechanical loading of neutrophil-muscle (C2C12)-cell co-cultures in the presence of superoxide dismutase (SOD) *(enzyme responsible for converting superoxide to hydrogen peroxide)* activated neutrophil cytotoxic activity and resulted in muscle cell lysis. The confirmation that MPO is the main origin was confirmed in MPO null mutant neutrophil (MPO-/-) and C2C12 co-cultures where no cell lysis was observed. This phenomenon was confirmed in an animal model as well, where a significant reduction (52%) in membrane lysis was observed in MPO-/- compared to wild type mice in response to reloading after a period of unloading (Nguyen *et al.* 2005). The previous chapter presented the data of subjects who resolved their muscle damage sooner than a second cohort who actually presented with additional leakage of indirect biomarkers of muscle damage following unaccustomed high intensity exercise. Furthermore an association between intramuscular MPO content and circulating indices of muscle damage *(creatine*

kinase (CK)) was demonstrated in these healthy young men *(refer to Fig. 5.12, chapter 5, p. 114)*.

In vitro cell lysis, *in vivo* membrane lysis and the association with leaking of CK suggest that the major role of excessive neutrophil activity targets the membrane. However in addition to the regained phagocytic activity, *in vitro* stimulation of isolated polymorphonuclear cells (PMN) with inflammatory mediators *(such as interleukin-8 (IL-8))* has been shown to result in the release of large quantities of matrix metalloprotease-9 (MMP-9) from neutrophilic tertiary granules (Chakrabarti *et al.* 2005, Ludwig *et al.* 1997, Ortega *et al.* 2009,2010, Pugin *et al*. 1999, Schlorke *et al.* 2012). MMP-9 plays a role in tissue destruction by degrading extracellular matrix components (Ohbayashi *et al.* 2002, Van den Steen *et al.* 2002). In professional male athletes, a statistically significant association was observed between increases in serum MPO, MMP-9 and interleukin-6 (IL-6) concentrations following incremental cycling to exhaustion (Reihmane *et al.* 2012). This study implied that the serum measures represented the localised response in the injured niche. However, the exercise did not contain an eccentric component. Therefore, despite the overwhelming evidence implicating neutrophils in causing damage to healthy tissue adjacent to the injury zone, a definitive cause-effect relationship in humans remains to be elucidated.

One of the important findings of the previous chapter was that the appearance of higher levels of IL-6 in circulation and a statistically significant elevation in intramuscular MPO was only evident in individuals who experienced secondary skeletal muscle damage *(refer to chapter 5, p. 98)*. All of the individuals did however have a similar increase in circulating leukocytes following the unaccustomed exercise. In a recent review Tidball *et al.* (2010) emphasised that both neutrophils and classically activated macrophages amplify the inflammatory response by producing inflammatory cytokines.

Interleukin-6 high and low responders following 2.5 hours of treadmill running *(75% VO_2max)* were previously identified (Ostrowski *et al.* 1998). Although regular measurements of indirect markers of muscle damage were taken over a period of 5 days, the authors did not investigate the individual CK responses during the late recovery phase. In the subjects who were classified as IL-6 high responders, increases in serum IL-6 were accompanied by a rapid anti-inflammatory cytokine response. A similar anti-inflammatory *(interleukin-10 (IL-10))* response was observed in all individuals during our previous investigation, but it was short-lived *(refer to chapter 5, p. 98)*. It is therefore not clear what the role of IL-6 might be in an

excessive secondary damage response. Only further analysis of immune cell status and the intramuscular inflammatory response could possibly shed light on this issue.

Reductions in resting serum cytokine concentrations in sedentary young men following six weeks of intense airforce training were related to decreased peripheral blood mononuclear cell (PBMC) NFκB gene expression (Sousa e Silva *et al.* 2010). In agreement, Jimenez-Jimenez *et al.* (2008) demonstrated that a significant increase in p65/p50 activity and the phosphorylation of IκBα in PBMC's following an acute bout of eccentric exercise was reduced with 8 weeks of eccentric training and a systemic inflammatory response prevented. Based on the evidence from these training studies involving exercise-induced muscle damage (EIMD) we hypothesized that PBMC NFκB signalling might be involved in the production of IL-6 following the cessation of exercise and that the extent may be indicative of a predisposition to secondary muscle damage.

The objectives of this study therefore included the evaluation of circulating neutrophil and macrophage functional responses and the association of related parameters with the second phase of skeletal muscle damage following high intensity intermittent downhill running. A secondary objective of this study was to evaluate the interaction between pro- *(IL-6, IL-8)* and anti- *(interleukin-1 receptor antagonist (IL-1ra), interleukin-4 (IL-4), IL-10)* inflammatory cytokine responses and their association with each category with PBMC NFκB signalling.

6.2 Methodology

For in depth information on methodology and laboratory protocols refer to chapter 4.

6.2.1 Subjects

Eighteen healthy young men volunteered to participate in this study and were randomly subdivided into a control (n=5) and downhill run (DHR) group (n=13). All of the participants were unaccustomed to any form of downhill activity and have never before volunteered to participate in any research study. This new group of participants was informed of the purpose and risks of the study before signing an informed consent form. The experimental protocol had been approved by the Committee for Human Research at Stellenbosch University and the

study was conducted according to the ethical guidelines and principles of the International Declaration of Helsinki.

6.2.2 Exercise testing

6.2.2.1 Incremental VO_2max test

Following a 5 minute (min) warm-up, all participants were asked to wear an oxygen mask linked to a metabolic system *(Oxycon Pro, Jaeger, Germany)* for breath-by-breath gas analysis. The incremental fitness test to exhaustion started at 8 $km.h^{-1}$ on a level treadmill (*Runrace, Technogym, Italy*), whereafter the speed was increased by 0.5 $km.h^{-1}$ every 30 seconds.

6.2.2.2 Downhill run

The participants in the DHR group performed a 60-min intermittent downhill run *(12 x 5 min bouts @ 15 $km.h^{-1}$, 10% decline)* on a motorized treadmill (*Runrace, Technogym, Italy*). Participants were allowed a 5 min standing rest between bouts and all the participants were able to complete all twelve bouts.

6.2.3 Blood sampling

Venous blood was drawn from the antecubital vein in the supine position before *(pre)*, immediately post, 2 hours (h) post and 4h post DHR on day 0 and on days 1 ,2, 3 and 4. Whole blood (*EDTA tubes*) samples collected on day 0 were analysed using flowcytometry (*FACS Canto, BD Biosciences*) for multiple intracellular and surface markers after multicolour fluorescent staining. The expression of intracellular myeloperoxidase (MPO) *(FITC Anti-human, #333138, BD Biosciences)* and cell surface CD11b/Mac1 *(Horizon V450 Anti-human, # 560480, BD Biosciences)*, CD163 *(APC Anti-human , #333610, Biolegend)*, CD88/C5aR *(PE Anti-human #344304, Biolegend)*, CD68 *(PerCPCy5.5 Anti-human, #333814, Biolegend)* and CD45 *(APCCy7 Anti-human, #557833, BD Biosciences)* were determined within the granulocyte and monocyte region of the flow cytometer scattergram. Total and differential white blood cell (WBC) counts were also determined using a universal cell counter (*Cell-Dyn 3700 Diagnostech).* Serum was analyzed using a one-step sandwich assay for creatine Kinase (CK) activity *(CARDIAC Calibrator # 386371)* and immunoassays

(Milliplex xMAP, Merck) in combination with the suspension array system *(Luminex 100)* was used to detect IL-1ra, IL-4, IL-6, IL-8, IL-10 *(Milliplex human cytokine/chemokines panel I HCYTOMAG-60K-05), sIL-6R (Milliplex human soluble cytokine receptor HSCR-32K-01), sICAM-1, sP-Selectin, MPO (Milliplex Human CVD panel 2, HCVD2MAG-67K-03), MMP-9 (Milliplex human MMP2 kit, HMMP2-55K-01).*

6.2.4 Peripheral blood mononuclear cell isolation

Peripheral mononuclear cells were isolated from whole blood collected in EDTA containing tubes on day 0 using density centrifugation and a cell separation medium *(Histopaque-1077, Sigma-Aldrich,#RNBB9926*).

Following isolation of PBMC's the samples collected at 2h post downhill running were immediately stained using fluorescent antibodies (*Rabbit polyclonal NFκB p65, abcam, #ab16502 ; Alexa Fluor 594, goat anti-rabbit, Invitrogen; bisBenzimide H33342 trihydrochloride, 099K400, #B2261-100MG*). Fluorescent microscopy was performed on an inverted fluorescent microscope *(Olympus IX81)* and images taken at x40 magnification *(Olympus LUCPlanFLN 40x/0.60 Ph2 ∞/0-2 FN22)* using camera *(F-view-II cooled CCD , Soft Imaging systems)*. The percentage of nuclei *(4'6-diamidino-2phenylindale, DAPI) (blue)* co-localized with NFκB p65 *(TexasRed) (red)* was determined for each cell using Manders coefficient (*Image J 1.43, Wayne Rasband, National Institute of Health, USA, rsb.info.nih@gov/ij*). Nuclear localization was determined for each participant and the average number of cells was 94.6 ±22.6.

6.2.5 Statistical analysis

All values are presented as mean ± Standard Error (SE). Software *(STATISTICA 10, StatSoft)* was used for all statistical analysis. Box-and Whisker plots were used to establish the presence of outliers defined as more than two standard deviations from the mean. These outlying data points were excluded from analysis and if a specific subject had an outlying value at baseline, all time points for that particular subject were excluded from analysis for that specific variable. To determine whether a group difference existed at baseline, a Kruskal-Wallis non-parametric test with Dunn's multiple comparison was performed. Repeated measures ANOVA with Fisher's LSD post hoc test was used to determine significant effects

of time, group or group x time. For a few specific variables an effect of time in one specific subgroup was analysed using a one-way ANOVA with Fisher's post hoc test *(particularly when control group values were non-detectable or extremely low)*. Relationships between variables were determined using Spearman's non-parametric ranked correlation analysis. The level of significance was accepted at $p<0.05$. The abbreviations that will be used in the results section for text, tables and graphs are: *$p<0.05$, **$p<0.01$, ***$p<0.001$ for effect of time within each group, #$p<0.05$, ##$p<0.01$, ### $p<0.001$ indicating significant group difference at same time point.

6.3 Results

6.3.1 Subject characteristics

The subject characteristics *(mean ± SE)* (n=18) were as follow: age 20.6 ± 0.3 yr, height 1.8 ± 0.1 m, weight 76.2 ± 2.1 kg, body mass index (BMI) 23.6 ± 0.4 $kg.m^{-2}$ and fitness level *(VO_2max)* 52.0 ± 1.3 $ml.kg.min^{-1}$.

6.3.2 Creatine kinase

No change in CK activity was evident in the control group (n=5) for the duration of the study, a significant group effect ($p<0.001$) was evident between the control group and the downhill run participants (n=13) from 2h to d2 (*refer to Fig. 6.1 panel A, p. 133*).

The participants in the downhill run group (n=13) were subdivided based on their individual CK activity over time, using the same criteria as previously described *(refer to chapter 5, section 5.3.3.1, p. 99).* One participant experienced an exaggerated CK response *(CK activity d4 6676 $u.L^{-1}$)* and was excluded from all further analysis. CK activity of fifty four percent of the remaining participants peaked *(1911 ± 398 $u.L^{-1}$) (mean ± SE)* ($p<0.001$) on d1 followed by a gradual decline and returned to values not significantly different from baseline on d3 *(499 ± 132 $u.L^{-1}$)*. These participants were grouped together as the DHR1 subgroup (n=7). The remaining forty percent of participants had an initial peak in CK activity on d1 *(1711 ± 367 $u.L^{-1}$)* ($p<0.001$), followed by evidence of a second elevation in CK on d3 ($p<0.01$) *(1380 ± 682 $u.L^{-1}$)* and d4 *(1994 ± 885 $u.L^{-1}$)* ($p<0.05$). These participants were grouped together as the DHR2 subgroup (n=6).

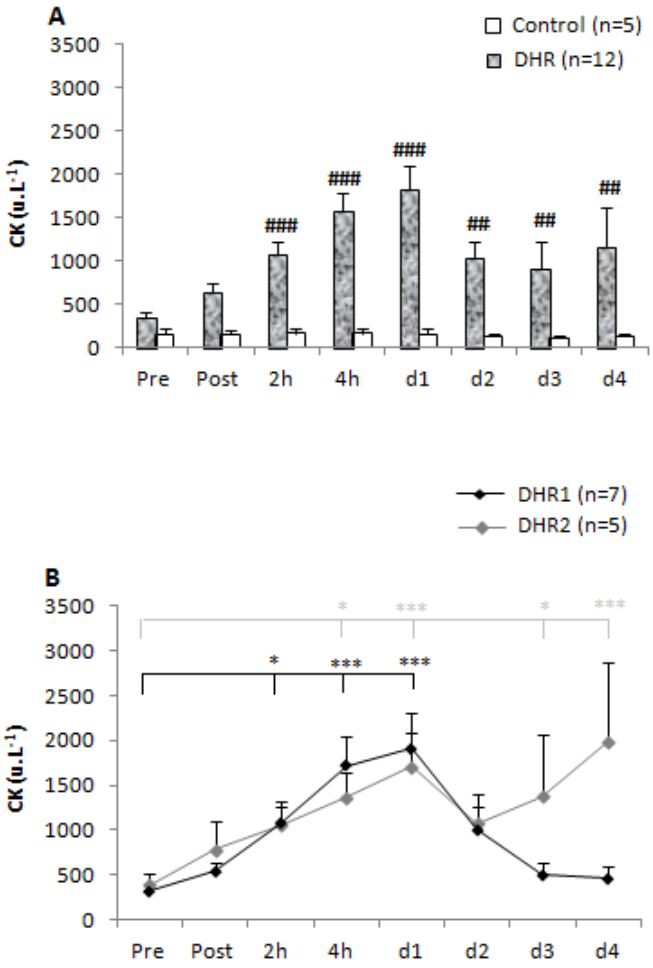

Fig 6.1 Creatine kinase activity *(mean ± SE)*. **Panel A** – DHR vs Control groups, **Panel B** – DHR1 vs DHR2 subgroups. **Statistical analysis:** Repeated measures ANOVA with LSD post hoc test. *p<0.05, **p<0.01, ***p<0.001 indicate significant differences from pre in each DHR subgroup. ##p<0.01 and ###p<0.001 indicate significant differences between the control and DHR group at the same time point. **Abbr:** CK- creatine kinase, d- day, h- hour.

6.3.3 Fatigue

The control group (n=5) did not experience any fatigue in the muscle groups of the lower limbs throughout the study. Both the DHR subgroups experienced moderate to severe overall fatigue (p<0.001), fatigue in quadriceps (p<0.001) and fatigue in calf muscles (p<0.001) immediately post downhill running and it lasted up to two days. The DHR2 subgroup

experienced a delayed moderate fatigue in the hamstrings that peaked on d1 (*refer to Fig. 6.2 Panel B, p. 134*).

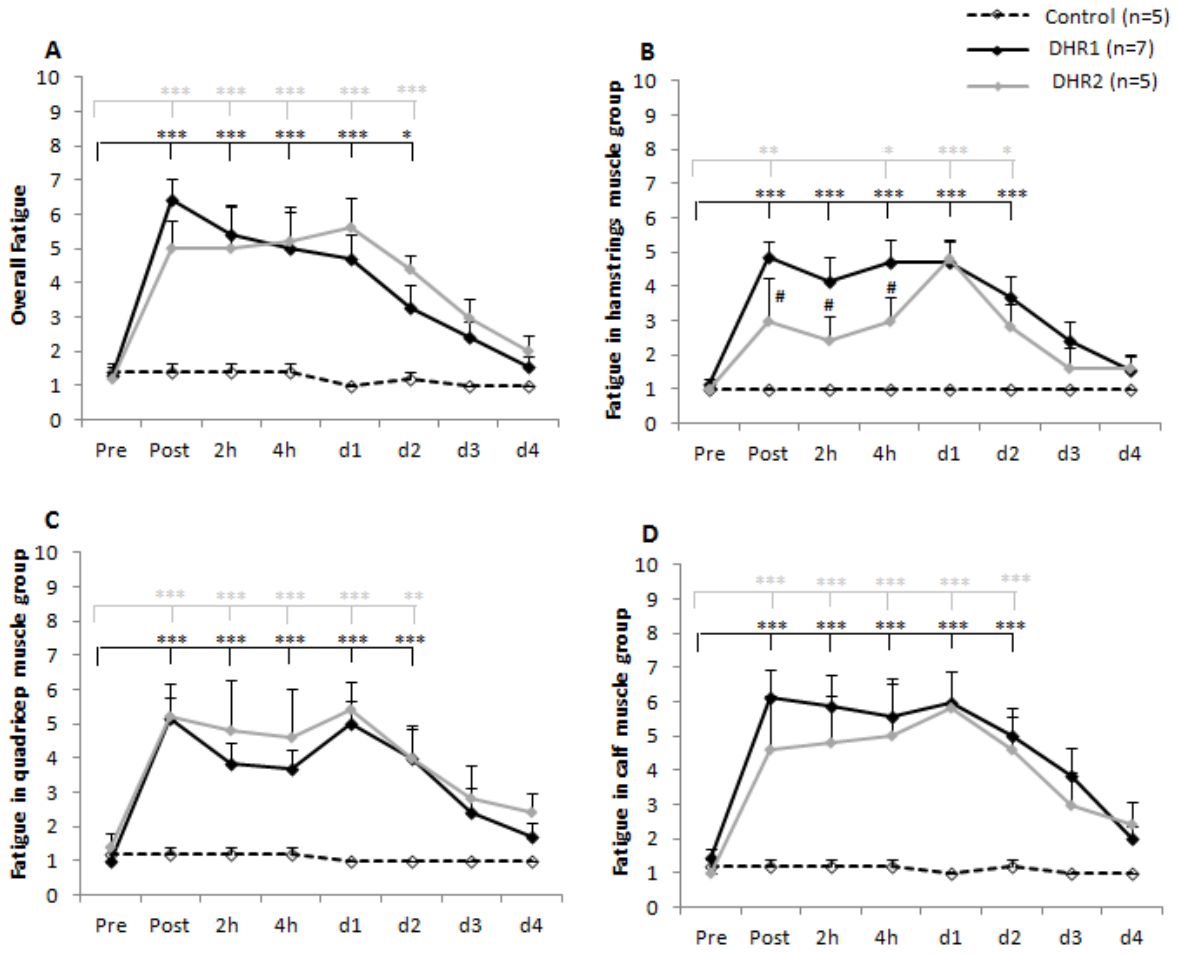

Fig 6.2 Fatigue (*mean ± SE).* **Panel A** – overall fatigue in legs, **Panel B** – fatigue in hamstring muscle group **Panel C** – fatigue in *quadriceps* muscle group **Panel D** – fatigue in calf muscle group. **Statistical analysis:** Repeated measures ANOVA with LSD post hoc test. *p<0.05, **p<0.01, ***p<0.001 indicate significant difference from pre in each DHR subgroup. #p<0.05 indicate significant difference between the DHR1 and DHR2 subgroup at the same time point. **Fatigue rating**: 1- none, 2- very slight, 3- slight, 4- mild, 5- moderate, 6- moderate to severe, 7- severe, 8- very severe, 9- very very severe, 10- totally exhausted. **Abbr:** d- day, h- hour.

6.3.4 Delayed onset muscle soreness (DOMS)

The control group (n=5) did not experience any muscle soreness in the quadriceps muscle group throughout the study. The DHR1 subgroup (n=7) experienced mild to moderately-severe muscle soreness in the quadriceps muscles immediately post downhill running that lasted for 2 days. In the DHR2 subgroup (n=5), muscle soreness in the *quadriceps* muscle

group at rest were delayed with moderate soreness experienced only after one day and lasted up to d3 following downhill running. A significant group effect was thus evident between the two DHR subgroups at 2h ($p<0.01$) and 4h ($p<0.05$). Similar to the DHR1 subgroup, the DHR2 subgroup experienced mild to moderately-severe muscle soreness immediately post downhill running when pressure were applied to the *quadriceps* muscles and when stretched. Muscle soreness during daily activities experienced on d2 ($P<0.05$) was rated higher in the DHR2 than DHR1 and remained above baseline on d3 only in the DHR2 subgroup (*refer to Fig. 6.3, below*).

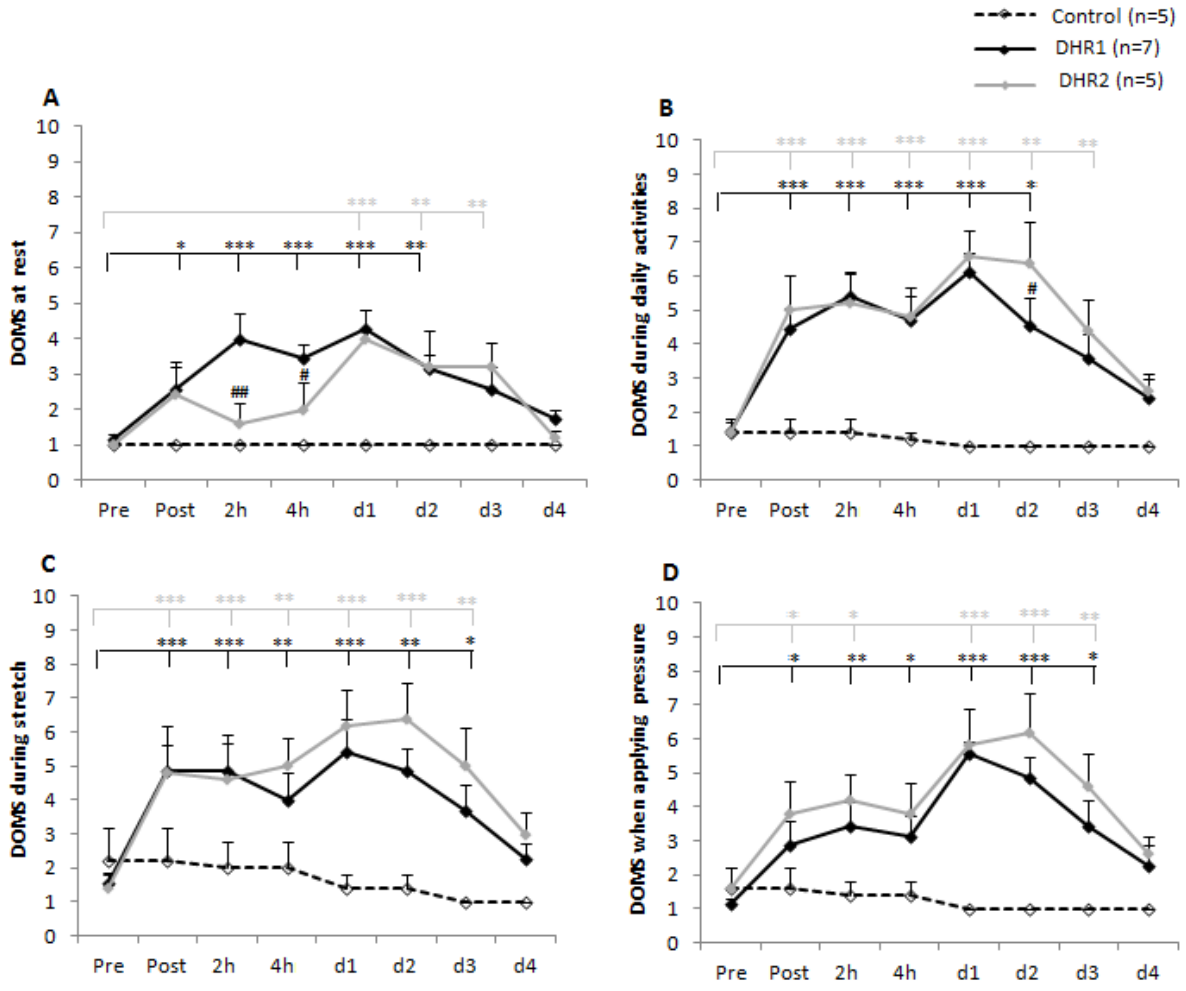

Fig 6.3. DOMS in *quadriceps* *(mean ± SE)*. **Panel A** – at rest, **Panel B** – during daily activities **Panel C** – during stretch **Panel D** – when applying pressure. **Statistical analysis:** Repeated measures ANOVA with LSD post hoc test. *p<0.05, **p<0.01, ***p<0.001 indicate significant difference from pre in each DHR subgroup. #p<0.05 and ##p<0.01 indicate significant difference between the DHR1 and DHR2 subgroup at the same time point. R**ating of perceived pain**: 1- no pain, 2- very slight, 3- slight, 4- mild, 5- moderate, 6- moderate to severe, 7- severe, 8- very severe, 9- very very severe, 10- unbearable pain **Abbr:** d- day, h- hour.

A highly significant *(r=0.9454, p=0.00003, n=12)* correlation was observed between the fatigue participants experienced in their hamstrings immediately post downhill running and the DOMS they experienced at rest 4 hours later (*refer to Fig. 6.4, below*).

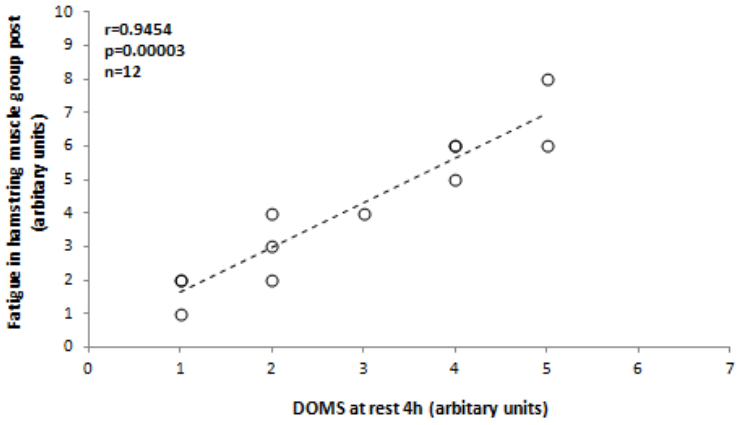

Fig 6.4. Fatigue in hamstrings (post) vs DOMS at rest (4h). **Statistical analysis:** Spearman's non-parametric correlation analysis, level of significance accepted at p<0.05. **Abbr:** DOMS – delayed onset muscle soreness, h- hour.

6.3.5 White blood cell (WBC) differential count

The total white blood cell (WBC) as well as differential WBC counts *(mean ± SE)* in the control group (n=5) remained within the normal clinical reference ranges *(total WBC 4.0-11.0 x10^{9} cells.L^{-1}; neutrophil 1.0-4.0 x10^{9} cells.L^{-1}; lymphocytes 1.0-4.0 x10^{9} cells.L^{-1}; monocyte 0.0-0.8 x10^{9} cells.L^{-1}; eosinophil 0.0- 0.4 x10^{9} cells.L^{-1}; basophil 0.0-0.1 x10^{9} cells.L^{-1})* throughout the study.

In the DHR1 subgroup (n=7), total WBC and neutrophil counts were significantly elevated immediately post downhill running with a peak observed at 4h *(WBC 12.6 ± 1.3 x10^{9} cells.L^{-1}; neutrophil 9.2 ± 1.1 x10^{9} cells.L^{-1})*, while monocyte count *(1.03 ± 0.07 x10^{9} cells.L^{-1})* increase was delayed with a significant elevation seen at 4h, which was also the peak. The DHR2 subgroup (n=5) also had significant increased WBC and neutrophil counts immediately post exercise, but had earlier peaks in total WBC *(14.5 ± 1.6 x10^{9} cells.L^{-1})* , neutrophil *(11.4 ± 1.8 x10^{9} cells.L^{-1})* and monocyte *(1.03 ± 0.02 x10^{9} cells.L^{-1})* counts appeared at 2h following downhill running. Total WBC and neutrophil count were thus

significantly different between the two DHR subgroups at 2h ($p<0.01$) (*refer to Fig. 6.5 Panel A and B, below*).

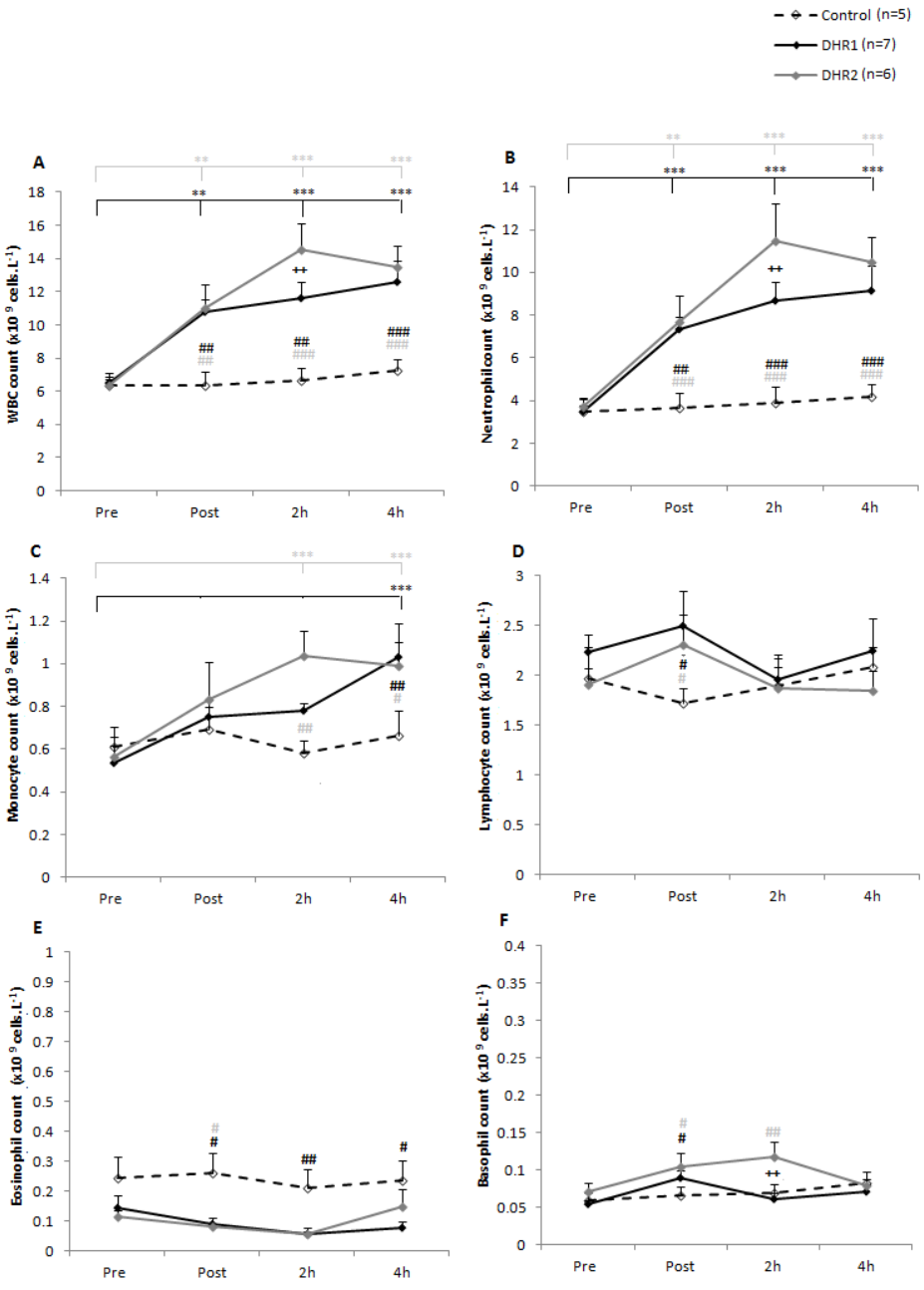

Fig 6.5 Differential white blood cell counts *(mean ± SE)*. **Panel A** – total WBC count, **Panel B** – Neutrophil count, **Panel C** - Monocyte count, **Panel D** - Lymphocyte count, **Panel E** – Eosinophil count**, Panel F** – Basophil count. **Statistical analysis**: Repeated measures ANOVA with LSD post hoc test. **p<0.01, ***p<0.001 indicate significant difference from pre in each DHR subgroup. #p<0.05, ##p<0.01, ###p<0.001 indicate significant difference between the control and DHR subgroup at the same time point. ++p<0.01 indicate significant difference between the DHR1 and DHR2 subgroup at the same time point. **Abbr:** WBC – white blood cell, h – hour, DHR- downhill run.

Since the main difference was seen at 2h, cell counts at this time point were correlated with CK activity and significant associations were found for total WBC count *(r=0.7090, p<0.05, n=10)*, neutrophil count *(r=0.6848, p<0.05, n=10)* and monocyte count *(r=8909, p<0.001, n=10)* when analysed individually. One source of the pro-inflammatory cytokines is considered to be immune cells and an association was found specifically between neutrophil count at 2h and IL-10 at the same time point *(r=0.9442, p=0.0004, n=10)* and between neutrophil count at 2h and IL-8 immediately post *(r=0.7698, p=0.015, n=9)* downhill running *(refer to Fig. 6.6, below)*

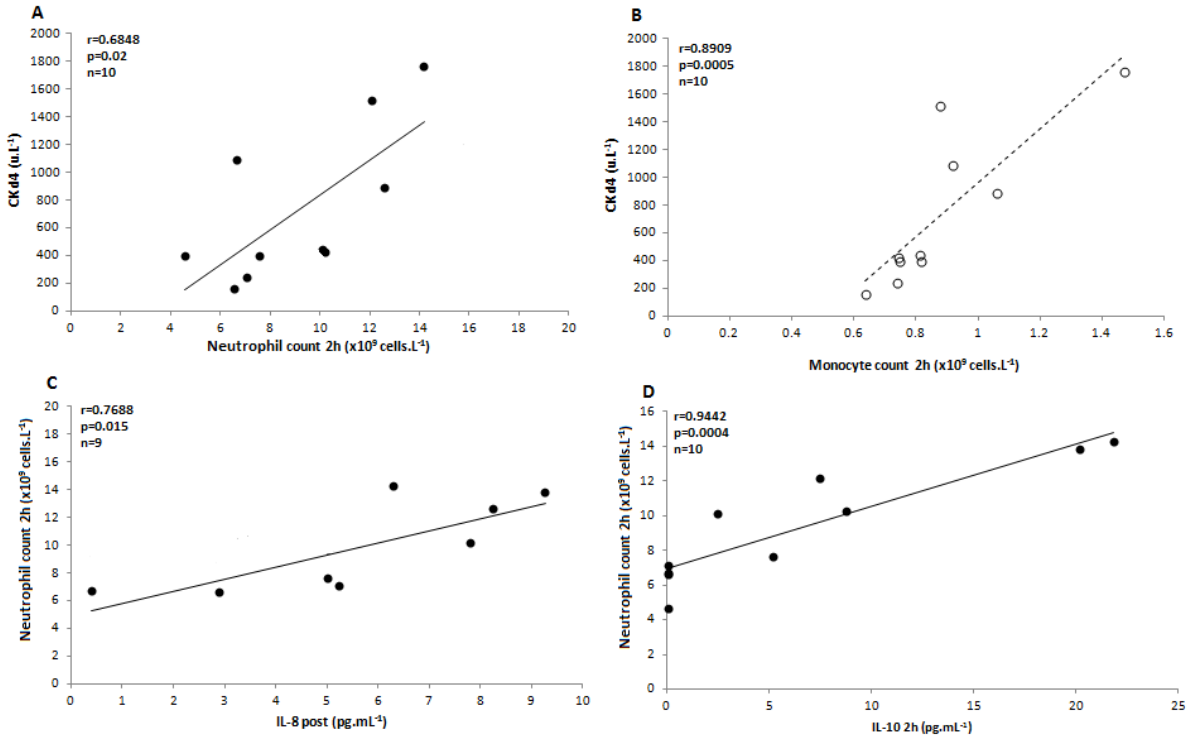

Fig 6.6. Spearman's correlation. Panel A – CK activity (d4) vs Neutrophil count (2h) **Panel B –** CK activity (d4) vs Monocyte count (2h) **Panel C –** Neutrophil count (2h) vs IL-8 (post) **Panel D –** Neutrophil count (2h) vs IL-10 (2h). **Statistical analysis:** Spearman's non-parametric correlation analysis, level of significance accepted at p<0.05. **Abbr:** CK- creatine kinase, d- day, h- hour.

6.3.6 Neutrophil activity

No change was observed in the surface protein CD11b or in the chemotaxic receptor ,C5aR on granulocytes in any of the subgroups in response to downhill running. Serum concentrations of soluble ICAM-1 *(243 ± 182 ng.mL^{-1}, n=12)* and soluble P-Selectin *(96 ± 12 ng.mL^{-1}, n=12)* remained unchanged throughout the study. Granulocyte intracellular MPO was unchanged in both the control and DHR1 subgroup whereas a decline ($p<0.05$) was observed at 4h in the DHR2 subgroup (n=5) (*refer to Fig. 6.7 Panel A, below*). A gradual increase in serum MMP-9 concentrations peaking at 4h *(DHR1 9845 ± 2638 pg.mL^{-1}, DHR2 10360 ± 5475 pg.mL^{-1})* was evident in both DHR subgroups which had returned to baseline by d1 (*DHR1 2966 ± 911 pg.mL^{-1}, DHR2 1872 ± 1267 pg.mL^{-1}*). Despite a similar response pattern in serum MMP-9 concentrations, statistical significance was only evident in the DHR2 subgroup *(refer to Fig. 6.7 Panel B, below),* most likely due to a lower pre-exercise MMP-9, rather than a different exercise-induced response.

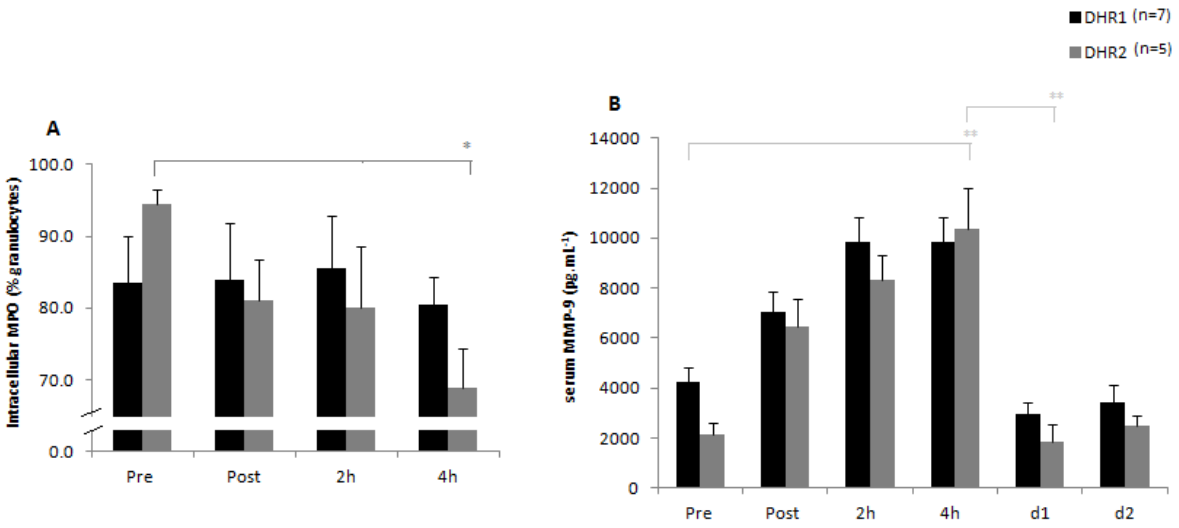

Fig. 6.7 Neutrophil activity. Panel A - Intracellular MPO *(granulocytes)*. **Panel B** –serum MMP-9. **Statistical analysis:** Repeated measures ANOVA with LSD post hoc test. *$p<0.05$, **$p<0.01$ indicate the effect of time within the DHR2 subgroup **Abbr:** d- day, h, hour, MPO- myeloperoxidase.

6.3.7 Monocyte/ macrophage activity

No change was observed in the surface protein of CD11b on monocytes/macrophages or in the percentage of circulating monocytes/macrophages containing intracellular MPO. A greater percentage of circulating macrophages ($CD68^+$) was evident in the DHR2 subgroup (n=5) immediately and 2h post ($p<0.05$) downhill running compared to the DHR1 subgroup (n=7) which did not change at all *(refer to Fig. 6.8 panel A, below)*. At 2h following downhill running more circulating anti-inflammatory macrophages ($CD163^+$) ($p<0.01$) were evident in the DHR2 compared to the DHR1 subgroup *(refer to Fig. 6.8 panel B, below)*. In terms of the percentage of monocytes this change was small, but in comparison to the DHR1 group it was significant.

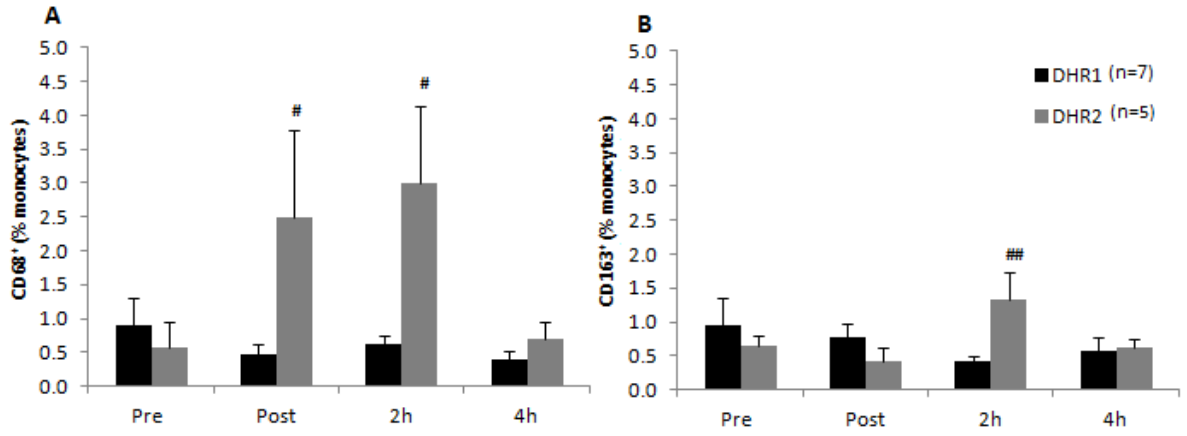

Fig. 6.8 Monocyte/macrophage activity. Panel A – CD68. **Panel B** –CD163. Statistical analysis: Repeated measures ANOVA with LSD post hoc test. #p<0.05, ##p<0.01 indicate significant difference between the DHR1 and DHR2 subgroup at the same time point. **Abbr:** h- hour.

6.3.8 p65 subunit of NFκB

The NFkB complex contains a p65 subunit and total NFκB was quantified by the intensity of a p65 bound fluorochrome *(refer to Fig. 6.9 panel B, p. 141)*. A proportion of p65 appears in the nucleus when NFκB is activated and the intensity of the p65-bound fluorochrome that co-localized with DAPI was expressed as a percentage of the total *(refer to Fig. 6.9 panel C, p. 141)*. This immunofluorescent miroscopy was done only at the 2h time point. Nuclear localization is high regardless of exercise *(control vs DHR1 subgroup)*. However a lower

percentage of nuclear localization was observed 2h following downhill running in the DHR2 subgroup. *(refer to Fig 6.9 panel E, below).*

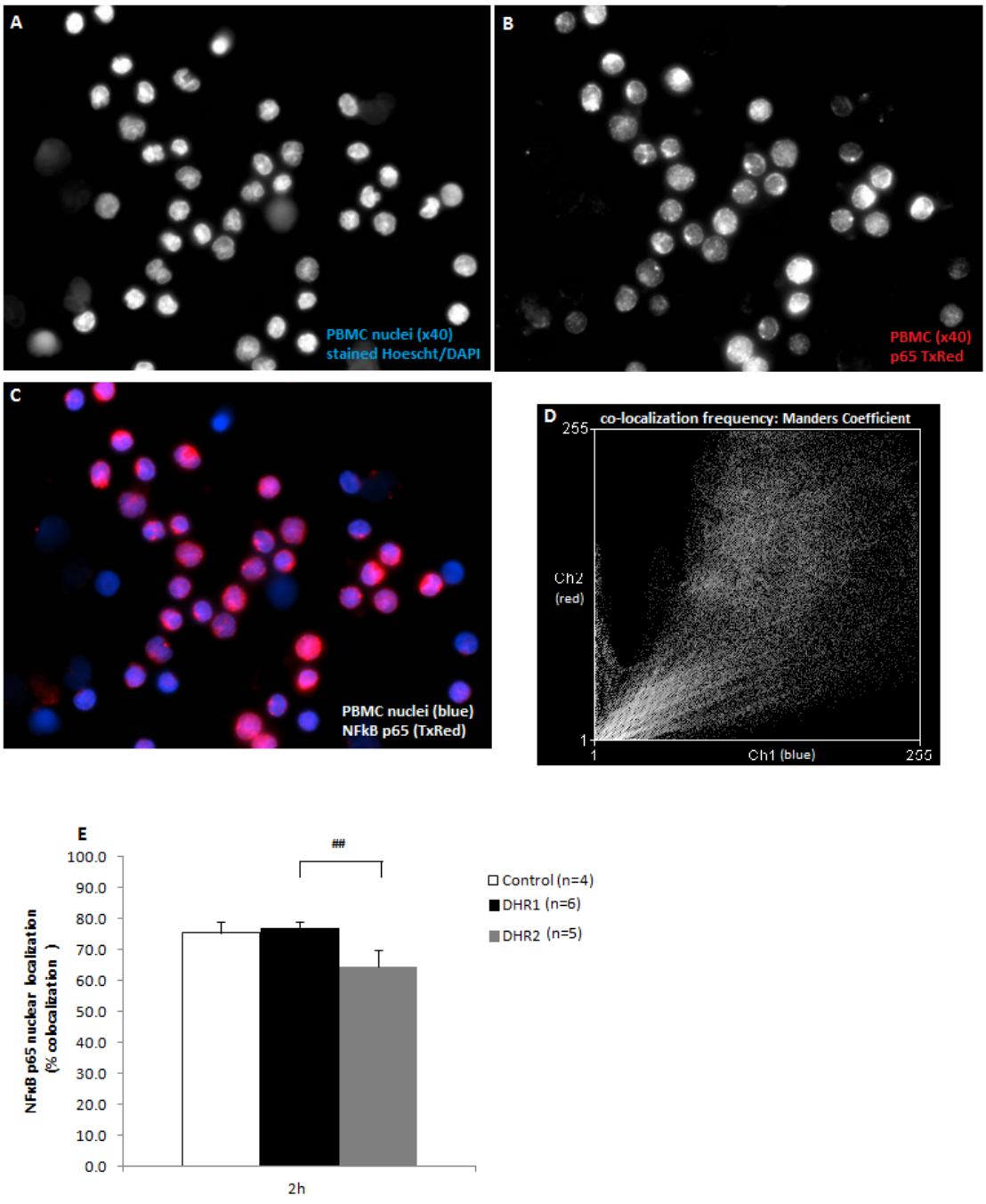

Fig. 6.9 NFκB signalling in PBMC's at 2h following DHR. Panel A – PBMC nuclei (Hoescht). **Panel B** – NFκB p65. **Panel C** - Merged image. **Panel D** – co-localization frequency. **Panel E –** Percentage of NFκB p65 nuclear localization *(mean ± SE)*. **Statistical analysis**: Co-localization was determined using Mander's coefficient in ImageJ software. Mann Whitney non-parametric with Dunns multiple comparison test. ## $p<0.01$ indicate significant difference between the DHR subgroups at the same time point. **Abbr:** h- hour

6.3.9 Cytokine response

No effect of time was observed in the serum cytokine concentrations *(IL-1ra, IL-4, IL-6, IL-10, sIL-6R)* in response to downhill running in the DHR1 subgroup. At 2h, serum IL-8 concentrations were slightly higher ($p<0.05$) compared to immediately post and d1 post downhill running in the DHR1 subgroup. These values did however not differ from baseline (pre) at any time *(refer to Fig. 6.10 panel A, p. 143)*.

A significant increase in serum IL-1ra, IL-6 and IL-10 concentrations were however evident in the DHR2 subgroup. Interleukin-1ra peaked *(23.6 ± 8.8 $pg.mL^{-1}$)* ($p<0.001$) at 2h followed by a gradual return to baseline. Interleukin-6 peaked at 2h *(3.6 ± 2.1 $pg.mL^{-1}$) (mean ± SE)* ($p<0.05$) and returned to baseline on d1 *(2.5 ± 1 $pg.mL^{-1}$)* whereas IL-10 peaked immediately post *(28.5 ± 10.2 $pg.mL^{-1}$)* ($p<0.001$) downhill running and returned to baseline at 4h *(2.8 ± 2.7 $pg.mL^{-1}$)*. This resulted in a significant difference between the DHR subgroups immediately post in serum IL-10 ($p<0.01$) concentrations and at 2h post downhill running in serum IL-10 ($p<0.05$), IL-6 ($p<0.05$) and IL-1ra ($p<0.01$) concentrations. A highly significant ($p<0.001$) group effect was also evident between the DHR subgroups in the ratio between serum IL-6 and IL-10 concentrations at 4h *(refer to Fig. 6.10 panel B, p. 143)*.

Despite a slight increase in serum sIL-6R concentrations immediately post downhill running in the DHR2 subgroup, statistical analysis indicated no effect of time, group or group x time. Serum IL-4 concentrations remained unchanged throughout the study with no effect of time, group or group x time evident.

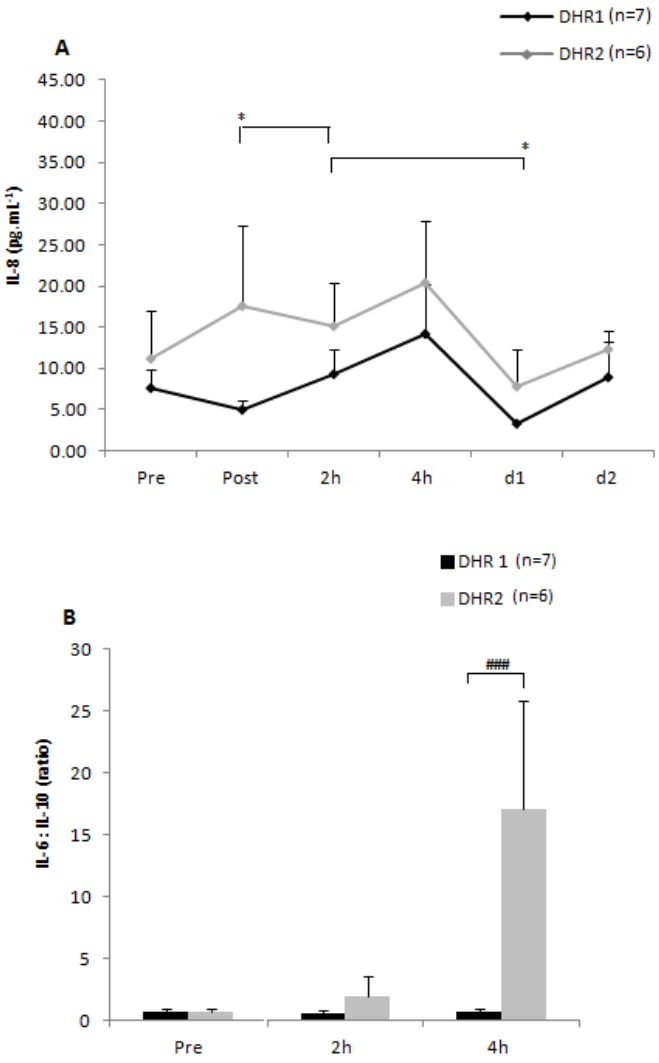

Fig. 6.10 Serum cytokines *(mean ± SE)*. **Panel A –** IL-8. **Panel B** – IL-6:IL-10 ratio. **Statistical analysis**: Repeated measures ANOVA with LSD post hoc test. *p<0.05 indicate effect of time within the DHR1 subgroup. ### p<0.001 indicate significant difference between the DHR subgroups at the same time point. **Abbr:** d- day, h- hour, IL- interleukin.

6.4 Discussion

This study confirms our previous findings, demonstrating that healthy young individuals respond differently to unaccustomed high intensity downhill running *(refer to chapter 5, p. 92)*. The advantages of this second study were: a) that a new cohort of subjects was recruited, b) the addition of a blood draw at 2h, c) the assessment of more parameters *(IL-1ra, IL-4, sIL-6R)* relating to a potential damping of the pro-inflammatory response, d) basic phenotyping of the circulating monocytes, and e) intramuscular MPO and NFκB analysis in

the previous study was not repeated, instead assessed in serum and whole blood subpopulations respectively.

Numerous related exercise studies have previously reported an association between changes in neutrophil count and creatine kinase activity (Ostapiuk-Karolczuk *et al.* 2012, Peake *et al.* 2005, Suzuki *et al.* 1999, Umeda *et al.* 2008). Both concentric and eccentric exercise induce leukocytosis *(refer to chapter 2, table 2.1, p. 27*). Natale *et al.* (2003) compared the effect of three different types of exercise on blood leukocyte count. A circuit of resistance exercise *(3x10 repetitions at 70% 1RM)* resulted in the largest changes in markers of muscle damage *(CK),* but prolonged aerobic exercise *(cycling at 60% VO_2max for 2 hours)* yielded the highest increase in total leukocytes. The main focus of most studies has been on events occurring during the early recovery phase and none investigated a late-onset secondary skeletal muscle damage. In addition to confirming the two different response patterns, the main finding was that the extent of leukocytosis at 2h post downhill running was significantly related to the additional appearance of indirect markers of muscle damage *(CK)* in circulation during the late recovery phase *(d4)*.

Findings related to the repeated bout effect support the notion that the extent of leukocytosis is not determined by the extent of exercise-induced muscle damage. Smith *et al.* (1998) indicated that the circulating leukocyte response to repeated bouts of EIMD remain consistent, whereas the significant elevations in CK activity were reduced. The current observations indicate that despite a similar increase in CK activity during the early recovery phase *(up to d1)*, individuals who experienced a second phase of muscle damage *(DHR2 subgroup)*, mobilized significantly more neutrophils and monocytes soon after exercise. These data support an hypothesis that the immune response is not only a function of exercise and a result of EIMD, but that it also promotes a delayed second phase of damage.

Cytokines are major effectors and regulators of immune cells, as well as being part of the communication network between different tissues, immune cells and the endocrine system. The release of inflammatory cytokines into the circulation in response to exercise stimuli appear to be responsible for the mobilization of leukocytes. Yamada *et al.* (2002) investigated whether the increase in circulating neutrophils in response to acute exercise *(maximal treadmill exercise)* is affected by the interaction between the endocrine and immune systems. The authors demonstrated that plasma IL-6 concentration 1h post exercise was significantly correlated to the number of circulating neutrophils at 2h post exercise in professional winter

sports athletes. In contrast, Paulsen *et al.* (2005) were unable to demonstrate a correlation between the changes in the leukocyte number and the changes in the plasma concentration of IL-6 following high force eccentric exercise *(quadriceps muscle group)* in healthy men. The difference in exercise protocols between these studies might explain the controversy. The intervention protocol in the present study, combined high intensity treadmill running and eccentric muscle contraction. In agreement with Paulsen *et al.* (2005) a direct relationship between serum IL-6 concentration and leukocytosis was not demonstrated. However, this lack of association might be the result of undetectable values or small changes in IL-6 concentrations in many of the individuals, specifically in the DHR1 subgroup. A relationship was however observed between serum IL-8 concentration immediately post and neutrophil count 2 hours post downhill running. It is well known that IL-8 has chemo-attractant properties (Yue *et al.* 1994). To our knowledge this is the first study to demonstrate a direct association between IL-8 and the mobilization of neutrophils in response to acute high intensity exercise not involving exercise-induced asthma.

Furthermore, a greater percentage of circulating monocytes/macrophages with a pro-inflammatory profile ($CD68^{+}$) was evident in the DHR2 subgroup immediately and 2h post downhill running. This coincided with a greater increase in serum IL-6 concentrations, suggesting that these immune cells may have been synthesising and releasing IL-6. Compared to the control and DHR1 subgroup, less NFκB p65 nuclear localization was evident in PBMC's of individuals in the DHR2 subgroup at this particular time point. Circulating mononuclear cells are thus unlikely to be the source of IL-6 appearing in circulation following the cessation of exercise. An alternate possibility is that neutrophils are responsible for the release of IL-6 since an elevated neutrophil count is evident at the same time point.

In accordance, Reihmane *et al.* (2012) observed a strong association between serum IL-6, MPO and MMP-9 release in professional athletes in response to maximal exercise *(cycling to exhaustion).* Based on this significant relationship, the authors suggested that neutrophils might be the main source for these inflammatory markers. Investigating the effects of physical exercise *(maximal cycling)* on blood cell-derived microparticles *(indicative of cellular activation or damage)*, Chaar *et al.* (2011) demonstrated an increase in the plasma concentration of microparticles derived from polymorphonuclear cells (PMNs). The authors demonstrated that PMN-derived microparticles remained elevated for up to two hours post exercise in healthy men, but more important was the observation of a similar pattern in

plasma IL-6 concentrations. Taken together, it can be argued that circulating neutrophils might have been responsible for releasing additional IL-6 into circulation *(independent of muscle contraction)* in a subset of individuals *(DHR2 subgroup)*. A proposed explanation such as this, from two variables associated at a particular time point, cannot be conclusive. The association could equally be proposed to signify that IL-6 release from micro-damaged muscle may be stimulating mobilization from bone marrow or epithelial demargination.

Previously, soluble interleukin-6 receptor (sIL-6R) has been implicated in controlling the pattern of leukocyte recruitment following acute EIMD, since infiltrating neutrophils were demonstrated to shed sIL-6R from their cell surface (Hurst *et al.* 2001, Robson-Ansley *et al.* 2010). Robson-Ansley *et al.* 2010 reviewed the findings of studies that investigated the effect of various forms of acute exercise on plasma sIL-6R concentrations. Despite taking the type of exercise intervention into account, the outcome of the 18 studies reviewed were very diverse. Forty four percent of these studies demonstrated no change (Depner *et al.* 2008, Gray *et al.* 2006, 2009, Keller *et al.* 2001, Robson-Ansley *et al.* 2009, Sacheck *et al.* 2006, Walshe *et al.* 2009), 22% indicated a decrease (Adamopoulos *et al.* 2002, Robson-Ansley *et al.* 2010, Smith *et al.* 2000, Zohlnhofer *et al.* 1992) and 33% observed an increase (Geiger *et al.* 2007, Keller *et al.* 2001, 2005, Patterson *et al.* 2008, Robinson *et al.* 2009, Robson-Ansley *et al.* 2006, Zohlnhofer *et al.* 1992) in plasma sIL-6R concentration. Two out of three studies involving EIMD however, showed an increase in plasma sIL-6R (Depner *et al.* 2008, Sacheck *et al.* 2006). Despite a slight tendency towards an increase, the present study was unable to detect any significant changes in serum sIL-6R concentration in either of the subgroups.

Like soluble IL-6 receptor, serum concentrations of soluble ICAM-1 and P-selectin result from shedding of these proteins, although not by immune cells themselves. Both remained unchanged and neither did surface expression of CD11b nor C5aR on neutrophils change in either of the subgroups. Increased plasma sICAM-1 and sP-selectin concentrations have been demonstrated on numerous occasions following marathon running (Nielsen *et al.* 2004, Parker *et al.* 2012, Reihmane *et al.* 2012). In contrast, despite an increase in PMN-derived microparticles, soluble forms of P-selectin, E-selectin, ICAM-1 and VCAM-1 remained unchanged post maximal cycling exercise (Chaar *et al.* 2011). Although the study by Chaar *et al.* (2011) did not involve EIMD, the observations are in accordance with those from the present study. Aikimoto *et al.* (2002) investigated the effects of various exercise interventions on circulating levels of sICAM-1 in healthy male subjects. The authors demonstrated an

increase in plasma sICAM-1 concentrations 1 day after endurance exercise *(42 km running)* , but not after concentric exercise *(bicycle ergometer, 80% VO2max)*. It would seem that the duration of weight bearing exercise may be a factor. However, the authors also observed an increase in sICAM-1 after downhill running *(30min @ ventilary threshold)* and suggested that changes in plasma sICAM-1 concentration may reflect the status of the immune system. Convincing evidence to support their findings are lacking. There are no other studies involving EIMD in healthy male subjects that have investigated changes in plasma sICAM-1 concentration.

There was however some evidence of neutrophil degranulation in the present study. Intracellular MPO content of neutrophils from the DHR2 subgroup decreased significantly, indicating that neutrophils released MPO into the circulation. Similarly, Peake *et al.* (2004) demonstrated that high intensity exercise *(60 min treadmill running, 80% VO_2max)* stimulated neutrophil degranulation, evident from a decrease in intracellular MPO content that coincided with an increase in plasma MPO concentrations. The authors furthermore demonstrated that neutrophil degranulation was accompanied by a slight decrease in the surface expression of CD11b.

In the current study, MMP-9, another component of neutrophilic tertiary granules, gradually increased in circulation peaking at 4h in both subgroups and returned to non-significant values by d1. Since no group effect was evident it is unlikely that MMP-9 cleavage was responsible for compromising sarcolemmal integrity during the late recovery phase. Koskinen *et al.* (2001) investigated the release of proteins with the ability to degrade extracellular matrix compounds *(including MMP-9)*, in healthy young men following downhill running. There was no association between MMP-9 release and CK activity. In the present study, the sharp decline in serum MMP-9 concentration from 4h to d1, coincided with the resolution of circulating leukocytosis. In addition to neutrophils, other leukocytes such as monocytes and lymphocytes can also synthesize and release MMP-9 (Chen *et al.* 2006). Our findings are therefore in agreement with the suggestion made by Koskinen *et al.* (2001), that the increase in serum MMP-9 concentrations reflect accelerated release of MMP-9 into circulation because of exercise-induced changes in leukocyte number rather than being associated with extracellular matrix breakdown that would be expected at later time points.

Chapter 5 demonstrated a close association between serum IL-6 concentrations immediately post downhill running and increases in serum IL-10 concentrations at the same time point.

The anti-inflammatory response *(serum IL-10)* was however short lived and had returned to baseline by 4h in all individuals *(refer to chapter 5)*. In addition to its association with IL-10, IL-6 is known to further induce an anti-inflammatory environment by stimulating the synthesis of IL-1ra (Ostrowski *et al.* 1998, Steensberg *et al.* 2003, Tilg *et al.* 1997). In the present study peak IL-6 concentrations were higher in the DHR2 subgroup and somewhat surprisingly, the anti-inflammatory responses were also higher *(IL-1ra and IL-10)*.

Recently, Ostapuik-Karolczuk *et al.* (2012) aimed to clarify the cytokine sequence in response to intense exercise *(90 min running at 65% VO_2max)* in non-athletes. A prolonged *(up to 2 days)* pro-inflammatory response *(TNFα, IL-1β)* was related to muscle damage (CK), possibly due to the long duration of exercise in untrained subjects. Despite this finding, the authors demonstrated that exercise induced an early anti-inflammatory response *(IL-10, IL-4)*.

The initial release of pro-inflammatory IL-6 is countered rapidly by the simultaneous release of IL-10 during exercise although the IL-1ra response occurs 2h after. Interleukin-10 concentrations peaked immediately post downhill running and remained elevated to a lesser extent up to 2h. At this particular time point, serum IL-10 concentration was significantly related to circulating neutrophil count. Studying the effect of exercise *(intensive cycling, 64% Watts(max))* on leukocyte cytokine mRNA expression, Nieman *et al.* (2006) found that circulating leukocytes substantially contribute to changes in plasma concentrations of IL-10 and IL-1ra. Nieman *et al.* (2006) did not distinguish between the leukocyte subsets in their analysis of anti-inflammatory mRNA since the RNA was isolated from the total leukocyte population.

It is thought that macrophages have either a pro- or anti-inflammatory phenotype. A significant increase in the number of circulating monocytes/macrophages with an anti-inflammatory profile ($CD163^+$) was evident in the DHR2 subgroup, shortly after the peak in serum IL-10 concentration and coinciding with peak IL-1ra concentrations. The anti-inflammatory properties of IL-10 are most likely involved in this phenomenon, since IL-10 is known to induce the differentiation of monocytes into anti-inflammatory macrophages (Allavena *et al.* 1997, 1998).

At 4h post downhill running, the anti-inflammatory response were no longer evident, whereas IL-6 remained elevated. The ratio between serum IL-6 and IL-10 at 4h, taken together with the significant correlation between CK activity *(d4)* and neutrophil count *(2h)*, strongly suggest that individuals in the DHR2 subgroup were unable to counter act the pro-

inflammatory properties of IL-6. Future investigations should focus on the interaction between immune cell activity and cytokines in an attempt to identify the mechanisms involved.

6.5 Conclusion

This is the first study to provide direct evidence implicating immune cell activity in the development of a second phase of skeletal muscle damage in a subset of healthy young men following high intensity intermittent downhill running. The cytokine response, although complex and difficult to interpret seem to greatly influence not only the mobilization of leukocytes but also the degranulation of neutrophils.

A direct association between IL-8 immediately post and slightly delayed peak in neutrophil count, support a role for IL-8 in the mobilization of neutrophils. The extent of leukocytosis at 2h post high intensity downhill running together with elevated serum IL-6 concentrations following the cessation of exercise might be an early indication of the development of a second phase of skeletal muscle damage. Despite a significant anti-inflammatory response occurring almost immediately following EIMD, it was short-lived. The delayed release of IL-6 into circulation after the resolution of the anti-inflammatory response confirms our previous observations implicating IL-6 in the development of sustained inflammation.

Why certain individuals are predisposed to the development of sustained inflammation and subsequent secondary skeletal muscle damage remains unclear and warrants further investigation. Future studies should investigate the precise source of the inflammatory biomarkers and focus on the interaction between pro- and anti-inflammatory responses.

CHAPTER 7 : CONCLUSION/ FUTURE RESEARCH

In a recent review discussing the effect of different exercise modalities on the appearance of CK in circulation, Baird *et al.* (2012) suggested: " Greater muscle cell disturbance delays the appearance of a CK serum peak compared to less disruption. This may be linked to the time course of inflammation; however, evidence in the literature supporting this theory remains unclear."

There are at least three issues influencing our lack of understanding:

Firstly, although it is well known that eccentric muscle damaging exercise results in an inflammatory cytokine response, the mechanisms and consequences of the acute inflammatory response is mostly based on evidence from animal models with extensive damage. The influence of inflammatory mediators *(cytokines)* on immune cell activity has also been studied mainly *ex vivo*. Cell culture studies only partly mimic the *in vivo* situation and how applicable these findings are to exercise-induced injury in humans is still not understood. Secondly, many studies have investigated either markers of muscle damage or the inflammatory response. Even studies reporting both, have seldom attempted to resolve how the two processes are linked. The purpose and outcomes of the studies that investigated the circulating cytokine response to EIMD in humans *(refer to chapter 2, table 2.1, p. 27)*, are very diverse. The variety of exercise protocols utilized and diverse subject populations in these studies might account for at least some of the discrepancies. Thirdly, large variability, in indirect markers of muscle damage, especially serum creatine kinase (CK) activity, has led to the questioning of the validity of this marker (Chapman *et al.* 2008, Clarkson *et al.* 1986, Kuipers *et al.* 1994, Manfredi *et al.* 1991). Subsequently, individuals have been classified into two groups: *CK low responders* and *CK high responders* (Larsen *et al.* 2007, Paulsen *et al.* 2005, 2010). These groundbreaking studies suggest that CK activity is not invalid.

Paulsen *et al.* (2005) demonstrated an association between a reduction in force-generating capacity and circulating leukocytosis following high force eccentric exercise. Based on these observations, taken together with correlations observed between serum cytokines *(Granulocyte colony stimulating factor (G-CSF), interleukin-6 (IL-6))* and circulating leukocytosis, the authors concluded that local processes in the damaged muscle regulate the non specific immune response (Paulsen *et al.* 2005, 2010). In agreement, Suzuki *et al.* (2003)

and Yamada *et al.* (2002) have previously discussed muscle damage as a probable cause of the cytokine response and provided circumstantial evidence implicating a cause-effect relationship between cytokines and the mobilization of leukocytes after endurance type exercise.

However, the metabolic role of one of the main pro-inflammatory cytokines, IL-6, has started a new controversy i.e that post exercise IL-6 in circulation is not due to muscle damage. Supporting this argument, Hirose *et al.* (2004) indicated that despite a second bout of high-force eccentric exercise resulting in less muscle damage than the first bout, no significant reductions in the cytokine response or leukocytosis was evident. Adding to the "reasonable doubt" is the inter-individual variability. We hypothesised that a better understanding of differences between low or high responders to skeletal muscle damage *(CK activity)* may provide the key to a better understanding of the interactions between damage, inflammation and resolution. The two apparently opposing views may therefore be reconciled when studying low vs high responders.

In 2010, Paulsen *et al.* observed a biphasic increase in serum CK levels specifically in high responders following high intensity eccentric exercise. The authors concluded that the initial peak in CK may reflect increased permeability of the sarcolemma, caused by the exercise itself and/or accumulation of leukocytes whereas the second peak was due to myofiber necrosis. The authors furthermore proposed that a large increase in circulating CK levels *(above 10 000 u.L^{-1})* precedes necrosis and results in long-lasting suppression of force-generating capacity. Significant intramuscular leukocyte accumulation was evident in these high responders.

Coinciding with the work done by Paulsen *et al*. (2010), we observed a similar biphasic increase in indirect markers of muscle damage *(CK, myoglobin, lactate dehydrogenase (LDH))* in healthy young men following high intensity downhill running *(data was unpublished at that stage)*. None of these individuals experienced an exaggerated CK response within the range of rhabdomyolysis (van de Vyver *et al.* 2012). Therefore, for the first time, this thesis presents convincing data that healthy young individuals experiencing the normal range of EIMD (with no differences in the initial peak of CK activity), can also be divided into two groups that reflect two different delayed responses after the same insult. The majority of the thesis attempted to find mechanisms for this observation.

In the current investigation we were able to provide direct evidence implicating immune cell activity in the development of a second phase of skeletal muscle damage in the subset of healthy young men following high intensity intermittent downhill running. In addition to identifying divergent individual CK response patterns, we clearly demonstrated that healthy young individuals, exposed to muscle micro-damage can be divided into those with a adequate and those with a less efficient capacity to control post-damage inflammatory processes. Compared to previous investigations the individuals who participated in the current research had a very narrow range for VO_2max indicating that the observed phenomena were not related to differences in ability or fitness.

Another advantage is that the research presented in this dissertation consisted of three longitudinal non-cross over studies, each with new volunteers *(healthy young untrained men)* recruited and tested using the same downhill run intervention protocol. The first two studies that formed part of the investigation were, to our knowledge, the first to show that a delayed peak in serum IL-6 at 4 hours (h) post EIMD is not a consistent finding and that individuals who did not exhibit this response had no secondary increase in CK release. As mentioned above, this phenomenon was confirmed by recruiting and testing new volunteers in study 3. The cytokine response, although complex and difficult to interpret still seems to be a key role player in the development of secondary skeletal muscle damage.

A schematic representation *(based on data from the current investigations)* of the circulating response following high intensity downhill running in these subsets of individuals is presented in *figure 7.1 (p. 153)*. Comparing the circulating response following high intensity downhill running between individuals with a normal recovery *(DHR1 subgroup)* and those experiencing secondary damage *(DHR2 subgroup) (refer to Fig. 7.1 panel A and B, p. 153)* highlights the importance of taking multiple measurements at regular time points. With the addition of the 2h time point *(study 3)*, we were able to provide evidence implicating neutrophil activity not only in compromising sarcolemmal integrity during the late recovery phase *(d3-d4)* but also as possible source of inflammatory cytokines. A very important and novel finding of the current investigation was that neutrophils peaked at 2h in the DHR2 subgroup, and despite a decrease remained elevated at 4h before a return to baseline by d1. In contrast, neutrophils in the DHR1 subgroup increased more slowly and peaked at 4h with the cell count similar to that of the DHR2 subgroup at this particular time point, but with overall less mobilization. Taken together with the higher levels of intramuscular MPO *(indicative of neutrophil accumulation)* observed two days post EIMD *(refer to Fig. 7.2 panel A and B,*

p.154), this finding suggests that neutrophil extravasation into the muscle occurred shortly after exercise in the DHR2 subgroup (between 2h and 4h) while mobilisation was still in progress in DHR1. A weakness of the current research is that biopsies were not taken immediately post and 2h post exercise to relate the circulating and intramuscular responses at these early time points. Intramuscular STAT3 signalling downstream of IL-6 was however higher at 4h in individuals who experienced a second phase of skeletal muscle damage *(refer to Fig. 7.2 panel A and B, p.154)* and this was significantly related to the circulating IL-6 response.

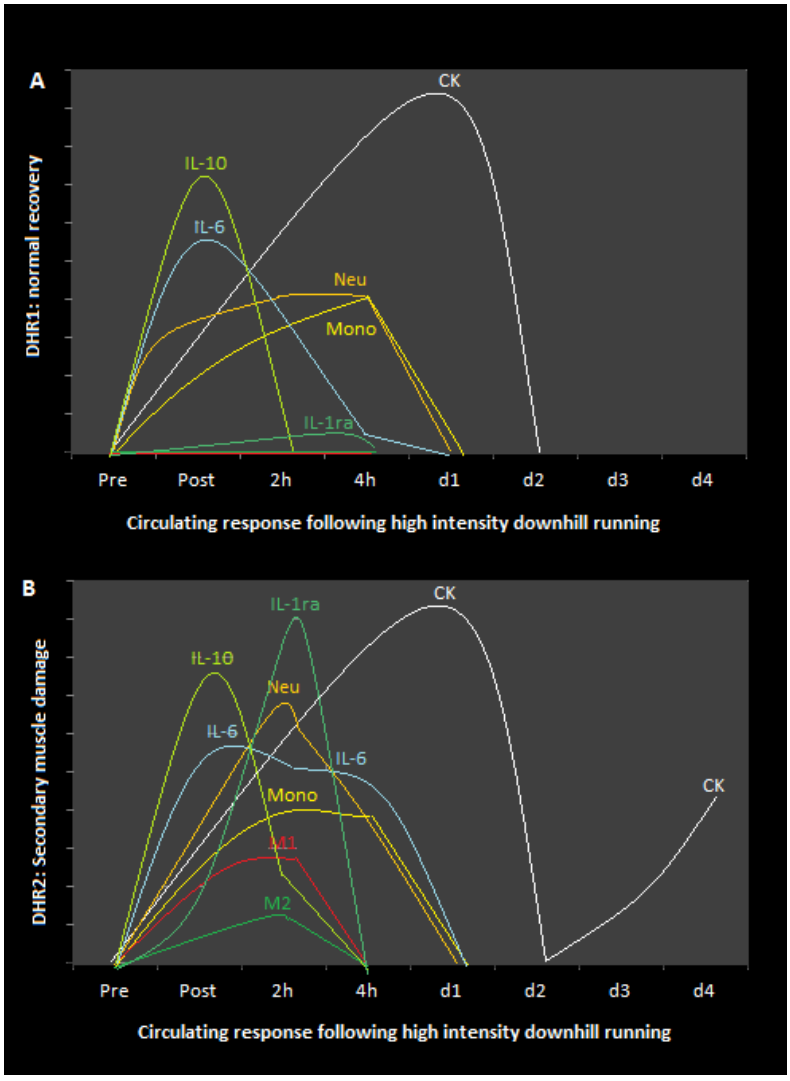

Fig. 7.1 Relative changes from baseline in the circulating response following high intensity downhill running in healthy young men. Panel A – DHR1 subgroup with normal recovery. **Panel B** – DHR2 subgroup experiencing secondary muscle damage. **Abbr:** CK – creatine kinase, d – day, h – hour, IL- interleukin, M1 – pro-inflammatory macrophages, M2 – anti-inflammatory macrophages, Mono – monocytes, Neu – neutrophils, ra – receptor antagonist.

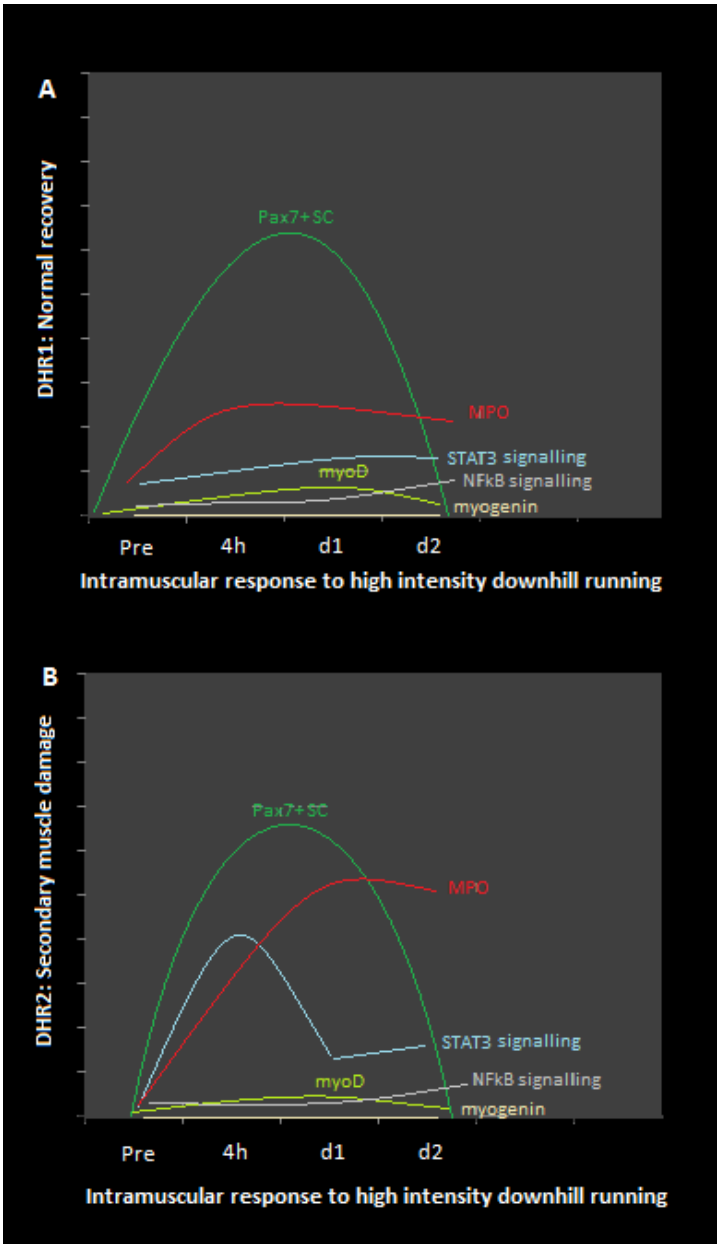

Fig. 7.2 Relative changes from baseline in the intramuscular response following high intensity downhill running in healthy young men. Panel A – DHR1 subgroup with normal recovery. **Panel B** – DHR2 subgroup experiencing secondary muscle damage. **Abbr:** d – day, h – hour, MPO – myeloperoxidase, SC – satellite cell, STAT – signal transducer and activator of transcription.

Coinciding with the initial increase in serum IL-6, a significant anti-inflammatory response occurred almost immediately following EIMD. This early rise in levels of anti-inflammatory cytokines, IL-10 and the soluble receptor, IL-1ra *(DHR2 subgroup only)* seemed to effectively suppress the synthesis/release of the major pro-inflammatory mediators such as TNFα and IL-1β. The anti-inflammatory response was however short-lived and no longer

evident in circulation at 4h post EIMD. The lack of IL-10 and IL-1ra in circulation at 4h suggest that the pro-inflammatory properties of IL-6 were no longer counter-acted.

The downstream effects of the circulating cytokine response during the first few hours following the cessation of exercise should be investigated further, since the events occurring during the early recovery phase seem to be indicative of events to follow during the late recovery phase.

Despite the development of relatively longer lasting inflammation in DHR2, the regenerative response seemed to be unaffected. A similar increase in the number of satellite cells expressing Pax7 was evident in all of the downhill running participants *(regardless of secondary damage)* . Myogenic regulatory factors involved in satellite cell cycle regulation and differentiation remained unchanged *(refer to Fig. 7.2 panel A and B, p. 154)*. It is possible that even though the downhill running intervention protocol resulted in severe muscle soreness and leakage of muscle proteins into the circulation, structural damage was insufficient to induce differentiation.

Why certain individuals are predisposed to the development of sustained inflammation and subsequent secondary skeletal muscle damage remains unclear and warrants further investigation. A number of correlations have been included in the thesis not because a cause-effect relationship can be accepted, but to provide suggestions for future studies. With studies designed to focus on particular observations more samples around the appropriate time points can be taken, rather than the full time course presented here.

CHAPTER 8: LIMITATIONS

The focus of the research presented in this dissertation was on the possible link between the individual differences in the inflammatory response and the later occurring indices of secondary damage. The research was thus explorative and no mechanistic relationships established. Most of the limitations to the research were associated with limited funding and/or the availability of facilities and materials. The following paragraphs will focus on some of these specific limitations.

There are numerous practical and logistical difficulties as well as ethical considerations to take into account when working with human subjects. One of which is the number of skeletal muscle biopsies that can be safely collected from a single subject without compromising his health. Taken together with the financial implications involved in skeletal muscle biopsy procedures it was necessary to limit the amount of biopsies taken per subject. Therefore, biopsies were only taken from 12 randomly selected subjects during the early recovery phase (4h, d1, d2) and 10 subjects during the late recovery phase (d3, d4). Similarly, serum myoglobin concentrations for instance could only be analyzed in a number of randomly selected subjects. These limitations taken together with the reliability of subjects lead to the analysis of samples from an inconsistent number of subjects for the different variables analysed. Furthermore, to maintain the statistical power of analysis the data from subjects who experienced an exaggerated and delayed peak in creatine kinase activity (DHR3) had to be excluded from all further analysis despite its potential importance. This was mainly due to low subject numbers in this particular subgroup. Data from the current investigation should however be used for a statistical power analysis to determine the number of subjects that need to be recruited for future studies to ensure an adequate distribution between the various DHR subgroups.

Since dehydration can exacerbate skeletal muscle damage, it was important to ensure that all participants were hydrated to a similar amount before their participation in the downhill running intervention protocol. Unfortunately, the current study did not have access to facilities with the capacity of measuring urine specific gravity. Instead the researchers relied on the haematocrit (%) as relative indication of the dehydration status of participants on the day of the intervention protocol. Future studies aiming to confirm the findings of the current investigation should however aim to accurately monitor the hydration status of participants

throughout the study. Despite all of the morphological and physiological responses that occur in response to exercise-induced muscle injury, one of the most important criteria is muscle function. Since the laboratories where the study was conducted did not have the capacity to measure the functional capacity of skeletal muscle, an arbitrary assessment of delayed onset muscle soreness and fatigue was used as an alternative. The repeatability of these measurements used in the current investigation can be questioned, especially since the outcome of the fatigue assessment were different between two studies *(presented in Chapter 5 and Chapter 6)* following the same intervention protocol. It is important to note though that the data obtained from the arbitrary assessment of fatigue and muscle soreness were merely used for descriptive purposes and did not form a key part in the outcome of the research project. Furthermore, serum sample analysis using either ELISA or bead array immune assays are also known to be variable. In order to provide credibility to results obtained from these specific assays samples should be analysed in duplicate. In the current investigation only the standard curve samples were however analysed in duplicate and not the experimental serum samples.

Key areas of interest identified in the current investigation should be confirmed in future studies with more in depth analysis focussing on a specific observation and the possible mechanisms involved.

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APPENDIX A: SUBJECT RECRUITMENT FLYER

WANT TO TAKE PART IN A RESEARCH PROJECT ?

We are looking for:

- ✓ Male athletes between the ages of 18 – 25
- ✓ Exercise between 2 and 6 hours per week
- ✓ Are not participating in hiking or hill walking or downhill-running
- ✓ Not being treated for or have a medical history of an inflammatory condition
- ✓ Have had NO muscle or exercise related injury in the previous 3 months.
- ✓ Do not suffer from any form of an infection or use of any prescribed or over the counter medication/ supplements

What will B done?

2x treadmill running tests

4x muscle biopsies

(x2 on each leg)

You may experience some pain and muscle soreness during the study…

Who will benefit ???

This study is done to better our understanding of inflammation and muscle regeneration after unaccustomed exercise and to broaden the scientific based evidence available for future reference.

Feedback will also given to participants :

Aerobic capacity (VO_{2max})

Fibre type composition

DEPARTMENT OF PHYSIOLOGICAL SCIENCES

Participants will be compensated for any inconvenience and their time for taking part in this study

For more information:

Contact

~~Mari 079 505 9708 /~~

~~15254127@sun.ac.za~~

APPENDIX B: PARTICIPANT INFORMATION LEAFLETS

B.1 INFORMED CONCENT FORM (ICF) – STUDY 1

NF*k*B signalling pathway responses to eccentric exercise and its involvement in a second phase of muscle damage.

The activity of an important messenger protein (NF*k*B) in the inflammatory pathway following downhill running and its association with a second phase of muscle.

PRINCIPAL INVESTIGATOR: Prof. Kathy Myburgh / Mari van de Vyver

ADDRESS: Exercise Physiology Laboratory (R2036)
Department of Physiological Sciences

Stellenbosch University

CONTACT NUMBER: 021 808 3146 / 079 505 9708

INFORMATION, POSSIBLE RISKS AND YOUR CONSENT

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details and possible risks of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied and that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the Committee for Human Research at Stellenbosch University and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

- The aim of the study is to investigate the activity of important messenger proteins (NF*k*B) inside the muscle that are associated with inflammation and can possibly cause additional muscle damage following exercise. Inflammation is a necessary response but if it is too much it can delay muscle repair.

- The knowledge gained from this study, could be beneficial to athletes participating in a variety of sports. New knowledge regarding the molecules inside the muscle which signal the presence of an inflammatory response to the unaccustomed exercise, could shed some light on the time course of events. This study will give an indication of when is the most suitable time for an athlete to seek help for recovery (following exercise induced muscle soreness), which could decrease the secondary damaging effects that delay muscle regeneration.

- The study will be conducted in the Exercise physiology laboratory at the Mike de Vries building, (Department of Physiological sciences), Stellenbosch University. The total number of 20 subjects will be randomly (drawing names from a hat) subdivided into an experimental (n=16) and control group (n=4).

- Feedback will be given to you following the exercise tests and laboratory analysis regarding your individual fitness level and muscle fibre type composition, this information can then be used by your coach to optimise your regular training program.

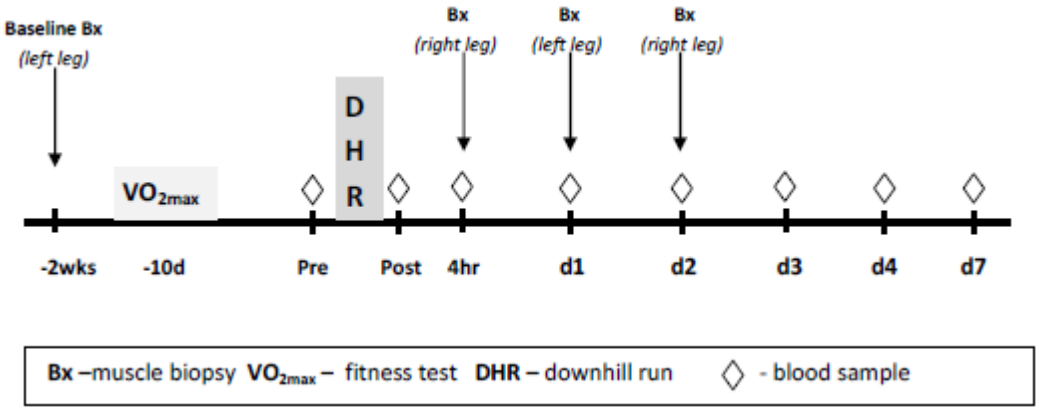
- Regardless if you are subdivided into the experimental or control group, you will be asked to maintain more or less the same amount of physical activity throughout the 3 weeks of the study and to keep a diary of your physical activity starting one week before the exercise (VO_{2max}) test and to continue up until the end of the study on day 7. It is necessary to record as accurately as you can because we need to know the daily variation of your training which can influence the messenger proteins in your muscle.

What will be done?

Subjects in the experimental group will participate in two exercise tests on a treadmill – first a baseline fitness (VO_{2max}) test and then a downhill run 10 days later. Subjects in the control group will only participate in the baseline fitness (VO_{2max}) test.

Blood samples (vein at your elbow) and muscle biopsies (from side of upper leg) will be collected at the same time points for everyone who participates in the study (both the experimental and control group). (*refer to fig below where the diamonds and arrows are marked).*

- **Study design indicating time course of experiment:**



- **Exercise testing:**

VO_{2max} is the maximal oxygen that your body is capable of using per minute to produce energy. An incremental exercise test to fatigue will thus be used to determine your fitness level. The baseline biopsy *(see more info below)* will be taken before the incremental test so that it doesn't influence your performance during the fitness test and to give you sufficient time to recover, before you participate in an exercise test. Your fitness level will therefore be determined 2-4 days following the baseline muscle biopsy procedure.

Upon arrival in the exercise laboratory you will be familiarized with the treadmill (Runrace, Technogym, Italy) and the incremental test explained to you verbally. Once you fully understand the exercise testing procedure and all your questions are answered satisfactory, you will participate in the incremental test to fatigue.

- **Incremental exercise test protocol (VO_{2max}) :**

Before the test, you will warm-up on the treadmill for approximately 5 minutes, running at any speed you feel comfortable with. After the warm-up, you will be asked to wear a light running heart rate monitor with chest band and an oxygen mask that is linked to the *Oxycon system* for analysing the amount of oxygen you are breathing in.

You will start running at a speed of 8 km/h (fast walking) on a level treadmill. The speed will then increase every 30 seconds by 0.5 km/h until you reach voluntary exhaustion. You will be encouraged verbally to continue for as long as possible, but you can stop the test at any time for any reason what so ever.

- **Downhill running intervention protocol :** (Experimental group only)

The subjects in the experimental group will participate in 12 bouts of 5 min each at a 10% downhill slope on a motorized treadmill (RUNRACE HC1200; Technogym, Italy). There will be a 2 min standing rest between each bout of exercise and the speed will be set at 15 km/h. The duration of the test will be 60 minutes.

During the week after the downhill run the subjects in the experimental group may experience generalised muscle tenderness and pain in the thigh and calf muscles associated with Delayed Onset Muscle Soreness as result of the exercise protocol.

- **Muscle biopsies** (Both groups will be subjected to identical days of biopsy sampling)

A qualified medical doctor (Dr J Brink) who is well trained in the procedure will perform the biopsies under sterile conditions. During the procedure you will be given local analgesia (injection) applied at the site to be biopsied in your upper leg. The injection of the analgesic may be accompanied by pain for a short period of time. Once the analgesia starts working the doctor will perform the biopsy using a sterile biopsy needle (sterilised at Stellenbosch hospital, Merriman Ave). The biopsy itself will be accompanied by a sensation of extreme pressure, but some pain will be experienced once the analgesia wears off. During the procedure a piece of approximately 50-100mg of muscle will be taken. This is a relatively small amount almost the size of the tip of a ballpoint pen. This procedure will not have a negative influence on your muscle function and your muscle will recover completely in a short period of time. Although pain may be present at the biopsy site during sports participation for a period of 1-2 days, it is not sufficient to limit sports participation in non-contact sport. You should refrain from sporting activity for 1 day after a biopsy, but are free to recommence participation on day 2 after a biopsy.

At the end of the study a total number of 4 muscle biopsies will have been collected from you. A baseline biopsy will be taken 2-4 days before the VO_{2max} test. The biopsy 4 hours post-intervention will be taken on the opposite leg. The biopsy on day 1 will be taken on the first leg used for the baseline biopsy and will be taken 3 cm below the first biopsy. The biopsy on day 2 will be taken on the opposite leg, 3 cm below the biopsy taken at 4 hours.

- **Blood sampling** (Both groups will be subjected to identical days of blood sampling)

All blood samples will be drawn from the forearm by a qualified phlebotomist (trained and certified to draw blood) using a double sided needle and a standard serum separating tube (SST).

On the day of the downhill run a sample will be drawn pre- , immediately and 4 hours post- the downhill run for the experimental group and in the control group (do not participate in DHR) the pre- and immediately post sample will be separated by 1 hour. On day 1, 2, 3, 4 and 7 one sample will be drawn every morning from subjects in both groups *(refer to study design)*.

The total amount of blood that will be drawn from you is thus 10 ml per sample time's 8 equals 80 ml.

- **Muscle soreness assessment**

You will be asked to self-asses your muscle soreness by answering a questionnaire based on a modified borg scale which have a rating from 1 to 10 where 1 is no pain and 10 is associated with unbearable pain. You will be asked to fill out a questionnaire before the downhill run as well as immediately post, 4hrs post and on days 1, 2 , 3, 4 and 7 .

- NO MEDICATION WILL BE GIVEN TO YOU DURING THIS STUDY. You will also be asked to restrain from using any pain killers or anti-inflammatory drugs during the study.

Why have you been invited to participate?

For the study to show any scientific significant results the people who participate has to be randomly selected but must also be more or less matched concerning age, gender, health and physical activity. Therefore all healthy male athletes between the ages of 18 and 25 who are not used to any downhill activity are invited to participate in this study.

What will your responsibilities be?

You would have to be reliable and punctual every day during the course of the study and **arrive at the exercise physiology lab on time**. To control the daily variations you would have to control your amount of exercise per week pre- and during the study, not exercising more than twice a week. You will be asked to keep a diary of your physical activity during the duration of the study.

Are there any risks involved in your taking part in this research?

- The possible risks involved in this study include normal injuries that can occur during exercise, e.g. muscle cramps, muscle soreness and micro-damage to the exercising muscles. This will however not be permanent and exercising muscles will heal completely within a couple of days. Despite the possible exercise related injuries, there is also a slight chance of an infection at the site of the muscle biopsy (less than 0.02% or 1 in 500) or some bruising at the site of blood sampling. In the unlikely event of such an injury occurring, you should immediately contact the study investigators or doctor.

If you do not agree to take part, what alternatives do you have?

Your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever.

Who will have access to your medical records?

- All information collected will be treated as strictly classified and only the study investigator and supervisor will have access to it. When the information is used in a publication or thesis the identity of all the participants will remain anonymous.

What will happen in the unlikely event of some form of injury occurring as a direct result of your taking part in this research study?

- In such an unlikely event of a serious injury as a direct result of the study protocol the department of physiological sciences, Stellenbosch University will offer compensation to the participant for any medical fees or other related costs involved for the participant.

Will you be paid to take part in this study and are there any costs involved?

You will be compensated pro rata for your time and any inconvenience for participating in the study but if you withdraw from the study at any point after you agreed to participate you will only be compensated for the part that you did participate in. There will be no additional costs involved for you, if you do take part.

Is there anything else that you should know or do?

- You can contact the department of physiological sciences at tel 021 8083149 if you have any further queries or encounter any problems.
- You can contact the Committee for Human Research at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor/investigator.
- You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I ……..……. agree to take part in a research study entitled: NF*k*B signalling pathway responses to eccentric exercise and its involvement in a second phase of muscle damage.

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) .....…..…….. on (*date*) ……...…….. 2010.

....

Signature of participant Signature of witness

Declaration by investigator

I *(name)* ……..…… declare that:

- I explained the information in this document to ……..
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use an interpreter. (*If an interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) ……...…..…….. on (*date*) ……...…….. 2010.

....

Signature of investigator Signature of witness

Declaration by interpreter

I *(name)* ……..…… declare that:

- I assisted the investigator (*name*) ……. to explain the information in this document to (*name of participant*) ……..…….. using the language medium of Afrikaans/Xhosa.

- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) .....…..…….. on (*date*) ……...…….. 2010.

....

Signature of interpreter Signature of witness

B.2 REVISED ICF 2011 – STUDY 2

NF*k*B signalling pathway responses to eccentric exercise and its involvement in a second phase of muscle damage.

The activity of an important messenger protein (NF*k*B) in the inflammatory pathway following downhill running and its association with a second phase of muscle.

PRINCIPAL INVESTIGATOR: Prof. Kathy Myburgh / Mari van de Vyver

ADDRESS: Exercise Physiology Laboratory (R2036)
Department of Physiological Sciences

Stellenbosch University

CONTACT NUMBER: 021 808 3146 / 079 505 9708

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What is this research study all about?

- The aim of the study is to investigate the activity of important messenger proteins (NF*k*B) inside the muscle that are associated with inflammation and can possibly cause additional muscle damage following exercise. Inflammation is a necessary response but if it is too much it can delay muscle repair.

- The knowledge gained from this study, could be beneficial to athletes participating in a variety of sports. New knowledge regarding the molecules inside the muscle which signal the presence of an inflammatory response to the unaccustomed exercise, could shed some light on the time course of events. This study will give an indication of when is the most suitable time for an athlete to seek help for recovery (following exercise induced muscle soreness), which could decrease the secondary damaging effects that delay muscle regeneration.

- The study will be conducted in the Exercise physiology laboratory at the Mike de Vries building, (Department of Physiological sciences), Stellenbosch University. The total number of 20 subjects will be randomly (drawing names from a hat) subdivided into an experimental (n=16) and control group (n=4).

- Feedback will be given to you following the exercise tests and laboratory analysis regarding your individual fitness level and muscle fibre type composition, this information can then be used by your coach to optimise your regular training program.

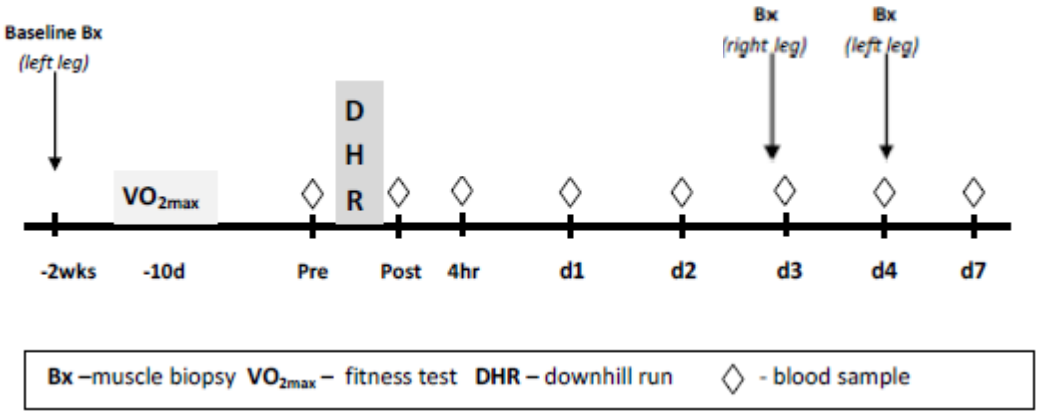
- Regardless if you are subdivided into the experimental or control group, you will be asked to maintain more or less the same amount of physical activity throughout the 3 weeks of the study and to keep a diary of your physical activity starting one week before the exercise (VO_{2max}) test and to continue up until the end of the study on day 7. It is necessary to record as accurately as you can because we need to know the daily variation of your training which can influence the messenger proteins in your muscle.

What will be done?

Subjects in the experimental group will participate in two exercise tests on a treadmill – first a baseline fitness (VO_{2max}) test and then a downhill run 10 days later. Subjects in the control group will only participate in the baseline fitness (VO_{2max}) test.

Blood samples (vein at your elbow) and muscle biopsies (from side of upper leg) will be collected at the same time points for everyone who participates in the study (both the experimental and control group). (*refer to fig below where the diamonds and arrows are marked).*

- **Study design indicating time course of experiment:**



- **Exercise testing:**

VO_{2max} is the maximal oxygen that your body is capable of using per minute to produce energy. An incremental exercise test to fatigue will thus be used to determine your fitness level. The baseline biopsy *(see more info below)* will be taken before the incremental test so that it doesn't influence your performance during the fitness test and to give you sufficient time to recover, before you participate in an exercise test. Your fitness level will therefore be determined 2-4 days following the baseline muscle biopsy procedure.

Upon arrival in the exercise laboratory you will be familiarized with the treadmill (Runrace, Technogym, Italy) and the incremental test explained to you verbally. Once you fully understand the exercise testing procedure and all your questions are answered satisfactory, you will participate in the incremental test to fatigue.

- **Incremental exercise test protocol (VO_{2max}) :**

Before the test, you will warm-up on the treadmill for approximately 5 minutes, running at any speed you feel comfortable with. After the warm-up, you will be asked to wear a light running heart rate monitor with chest band and an oxygen mask that is linked to the *Oxycon system* for analysing the amount of oxygen you are breathing in.

You will start running at a speed of 8 km/h (fast walking) on a level treadmill. The speed will then increase every 30 seconds by 0.5 km/h until you reach voluntary exhaustion.

You will be encouraged verbally to continue for as long as possible, but you can stop the test at any time for any reason what so ever.

- **Downhill running intervention protocol :** (Experimental group only)

The subjects in the experimental group will participate in 12 bouts of 5 min each at a 10% downhill slope on a motorized treadmill (RUNRACE HC1200; Technogym, Italy). There will be a 2 min standing rest between each bout of exercise and the speed will be set at 15 km/h. The duration of the test will be 60 minutes.

During the week after the downhill run the subjects in the experimental group may experience generalised muscle tenderness and pain in the thigh and calf muscles associated with Delayed Onset Muscle Soreness as result of the exercise protocol.

- **Muscle biopsies** (Both groups will be subjected to identical days of biopsy sampling)

A qualified medical doctor (Dr J Brink) who is well trained in the procedure will perform the biopsies under sterile conditions. During the procedure you will be given local analgesia (injection) applied at the site to be biopsied in your upper leg. The injection of the analgesic may be accompanied by pain for a short period of time. Once the analgesia starts working the doctor will perform the biopsy using a sterile biopsy needle (sterilised at Stellenbosch hospital, Merriman Ave). The biopsy itself will be accompanied by a sensation of extreme pressure, but some pain will be experienced once the analgesia wears off. During the procedure a piece of approximately 50-100mg of muscle will be taken. This is a relatively small amount almost the size of the tip of a ballpoint pen. This procedure will not have a negative influence on your muscle function and your muscle will recover completely in a short period of time. Although pain may be present at the biopsy site during sports participation for a period of 1-2 days, it is not sufficient to limit sports participation in non-contact sport. You should refrain from sporting activity for 1 day after a biopsy, but are free to recommence participation on day 2 after a biopsy.

At the end of the study a total number of 3 muscle biopsies will have been collected from you. A baseline biopsy will be taken 2-4 days before the VO_{2max} test. The biopsy on day 3 will be taken on the first leg used for the baseline biopsy and will be taken 3 cm below the first biopsy. The biopsy on day 4 will be taken on the opposite leg, 3 cm below the biopsy taken at baseline.

- **Blood sampling** (Both groups will be subjected to identical days of blood sampling)

All blood samples will be drawn from the forearm by a qualified phlebotomist (trained and certified to draw blood) using a double sided needle and a standard serum separating tube (SST).

On the day of the downhill run a sample will be drawn pre- , immediately and 4 hours post-the downhill run for the experimental group and in the control group (do not participate in DHR) the pre- and immediately post sample will be separated by 1 hour. On day 1, 2, 3, 4 and 7 one sample will be drawn every morning from subjects in both groups *(refer to study design)*.

The total amount of blood that will be drawn from you is thus 10 ml per sample time's 8 equals 80 ml.

- **Muscle soreness assessment**

You will be asked to self-asses your muscle soreness by answering a questionnaire based on a modified borg scale which have a rating from 1 to 10 where 1 is no pain and 10 is associated with unbearable pain. You will be asked to fill out a questionnaire before the downhill run as well as immediately post, 4hrs post and on days 1, 2 , 3, 4 and 7 .

- NO MEDICATION WILL BE GIVEN TO YOU DURING THIS STUDY. You will also be asked to restrain from using any pain killers or anti-inflammatory drugs during the study.

Why have you been invited to participate?

For the study to show any scientific significant results the people who participate has to be randomly selected but must also be more or less matched concerning age, gender, health and physical activity. Therefore all healthy male athletes between the ages of 18 and 25 who are not used to any downhill activity are invited to participate in this study.

What will your responsibilities be?

You would have to be reliable and punctual every day during the course of the study and **arrive at the exercise physiology lab on time**. To control the daily variations you would have to control your amount of exercise per week pre- and during the study, not exercising

more than twice a week. You will be asked to keep a diary of your physical activity during the duration of the study.

Are there any risks involved in your taking part in this research?

- The possible risks involved in this study include normal injuries that can occur during exercise, e.g. muscle cramps, muscle soreness and micro-damage to the exercising muscles. This will however not be permanent and exercising muscles will heal completely within a couple of days. Despite the possible exercise related injuries, there is also a slight chance of an infection at the site of the muscle biopsy (less than 0.02% or 1 in 500) or some bruising at the site of blood sampling. In the unlikely event of such an injury occurring, you should immediately contact the study investigators or doctor.

If you do not agree to take part, what alternatives do you have?

Your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever.

Who will have access to your medical records?

- All information collected will be treated as strictly classified and only the study investigator and supervisor will have access to it. When the information is used in a publication or thesis the identity of all the participants will remain anonymous.

What will happen in the unlikely event of some form of injury occurring as a direct result of your taking part in this research study?

- In such an unlikely event of a serious injury as a direct result of the study protocol the department of physiological sciences, Stellenbosch University will offer compensation to the participant for any medical fees or other related costs involved for the participant.

Will you be paid to take part in this study and are there any costs involved?

You will be compensated pro rata for your time and any inconvenience for participating in the study but if you withdraw from the study at any point after you agreed to participate you will only be compensated for the part that you did participate in. There will be no additional costs involved for you, if you do take part.

Is there anything else that you should know or do?

- You can contact the department of physiological sciences at tel 021 8083149 if you have any further queries or encounter any problems.
- You can contact the Committee for Human Research at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor/investigator.
- You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I ……..……. agree to take part in a research study entitled: NF*k*B signalling pathway responses to eccentric exercise and its involvement in a second phase of muscle damage.

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) .....…..…….. on (*date*) ……...…….. 2011.

....

Signature of participant Signature of witness

Declaration by investigator

I *(name)* ……..…… declare that:

- I explained the information in this document to ……..
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use an interpreter. (*If an interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) ……...…..…….. on (*date*) ……...…….. 2011.

....

Signature of investigator Signature of witness

Declaration by interpreter

I *(name)* ……..…… declare that:

- I assisted the investigator (*name*) ……. to explain the information in this document to (*name of participant*) ……..…….. using the language medium of Afrikaans/Xhosa.

- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) .....…..…….. on (*date*) ……...…….. 2011.

.....

Signature of interpreter Signature of witness

B.3 REVISED ICF 2012 – STUDY 3

NF*k*B signalling pathway responses to eccentric exercise and its involvement in a second phase of muscle damage.

The activity of an important messenger protein (NF*k*B) in the inflammatory pathway following downhill running and its association with a second phase of muscle.

PRINCIPAL INVESTIGATOR: Prof. Kathy Myburgh / Mari van de Vyver

ADDRESS: Exercise Physiology Laboratory (R2036)
Department of Physiological Sciences

Stellenbosch University

CONTACT NUMBER: 021 808 3146 / 079 505 9708

INFORMATION, POSSIBLE RISKS AND YOUR CONSENT

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details and possible risks of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied and that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the Committee for Human Research at Stellenbosch University and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

- The aim of the study is to investigate the activity of important messenger proteins (NF*k*B) inside the blood mononuclear cells that are associated with inflammation and can possibly cause additional muscle damage following exercise. Inflammation is a necessary response but if it is too much it can delay muscle repair.

- The knowledge gained from this study, could be beneficial to athletes participating in a variety of sports. New knowledge regarding the molecules inside whole which signal the presence of an inflammatory response to the unaccustomed exercise, could shed some light on the time course of events. This study will give an indication of when is the most suitable time for an athlete to seek help for recovery (following exercise induced muscle soreness), which could decrease the secondary damaging effects that delay muscle regeneration.

- The study will be conducted in the Exercise physiology laboratory at the Mike de Vries building, (Department of Physiological sciences), Stellenbosch University. The total number of 20 subjects will be randomly (drawing names from a hat) subdivided into an experimental (n=16) and control group (n=4).

- Feedback will be given to you following the exercise tests and laboratory analysis regarding your individual fitness level, this information can then be used by your coach to optimise your regular training program.

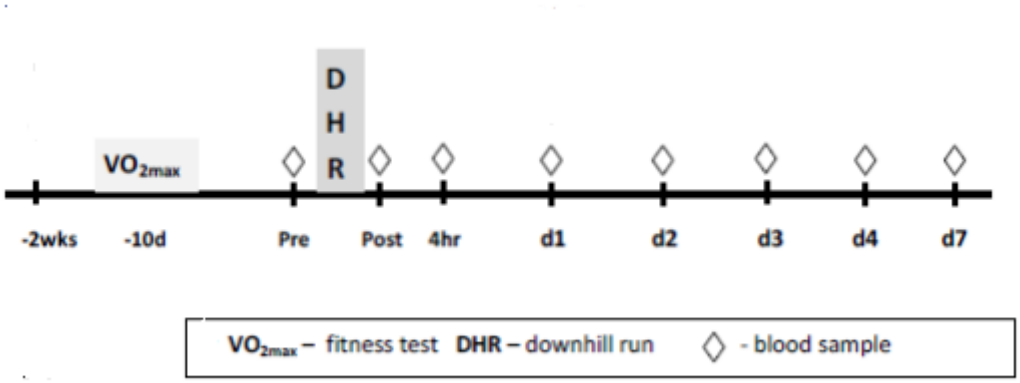
- Regardless if you are subdivided into the experimental or control group, you will be asked to maintain more or less the same amount of physical activity throughout the 3 weeks of the study and to keep a diary of your physical activity starting one week before the exercise (VO_{2max}) test and to continue up until the end of the study on day 7. It is necessary to record as accurately as you can because we need to know the daily variation of your training which can influence the messenger proteins in your muscle.

What will be done?

Subjects in the experimental group will participate in two exercise tests on a treadmill – first a baseline fitness (VO_{2max}) test and then a downhill run 10 days later. Subjects in the control group will only participate in the baseline fitness (VO_{2max}) test.

Blood samples (vein at your elbow) will be collected at the same time points for everyone who participates in the study (both the experimental and control group). (*refer to fig below where the diamonds and arrows are marked).*

- **Study design indicating time course of experiment:**



- **Exercise testing:**

VO_{2max} is the maximal oxygen that your body is capable of using per minute to produce energy. An incremental exercise test to fatigue will thus be used to determine your fitness level.

Upon arrival in the exercise laboratory you will be familiarized with the treadmill (Runrace, Technogym, Italy) and the incremental test explained to you verbally. Once you fully understand the exercise testing procedure and all your questions are answered satisfactory, you will participate in the incremental test to fatigue.

➢ **Incremental exercise test protocol (VO_{2max}) :**

Before the test, you will warm-up on the treadmill for approximately 5 minutes, running at any speed you feel comfortable with. After the warm-up, you will be asked to wear a light running heart rate monitor with chest band and an oxygen mask that is linked to the *Oxycon system* for analysing the amount of oxygen you are breathing in.

You will start running at a speed of 8 km/h (fast walking) on a level treadmill. The speed will then increase every 30 seconds by 0.5 km/h until you reach voluntary exhaustion. You will be encouraged verbally to continue for as long as possible, but you can stop the test at any time for any reason what so ever.

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The subjects in the experimental group will participate in 12 bouts of 5 min each at a 10% downhill slope on a motorized treadmill (RUNRACE HC1200; Technogym, Italy).

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The total amount of blood that will be drawn from you is thus 10 ml per sample time's 7 equals 70 ml.

- **Muscle soreness assessment**

You will be asked to self-asses your muscle soreness by answering a questionnaire based on a modified borg scale which have a rating from 1 to 10 where 1 is no pain and 10 is associated with unbearable pain. You will be asked to fill out a questionnaire before the downhill run as well as immediately post, 4hrs post and on days 1, 2 , 3, 4 and 7 .

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What will your responsibilities be?

You would have to be reliable and punctual every day during the course of the study and **arrive at the exercise physiology lab on time**. To control the daily variations you would have to control your amount of exercise per week pre- and during the study, not exercising more than twice a week. You will be asked to keep a diary of your physical activity during the duration of the study.

Are there any risks involved in your taking part in this research?

- The possible risks involved in this study include normal injuries that can occur during exercise, e.g. muscle cramps, muscle soreness and micro-damage to the exercising muscles. This will however not be permanent and exercising muscles will heal completely within a couple of days. Despite the possible exercise related injuries, there is also a slight chance of some bruising at the site of blood sampling. In the unlikely event of such an injury occurring, you should immediately contact the study investigators or doctor.

If you do not agree to take part, what alternatives do you have?

Your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever.

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- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) .....…..…….. on (*date*) ……...…….. 2012.

....

Signature of participant Signature of witness

Declaration by investigator

I *(name)* ……..…… declare that:

- I explained the information in this document to ……..
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use an interpreter. (*If an interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) ……...…..…….. on (*date*) ……...…….. 2012.

....

Signature of investigator Signature of witness

Declaration by interpreter

I *(name)* ……..…… declare that:

- I assisted the investigator (*name*) ……. to explain the information in this document to (*name of participant*) ……..…….. using the language medium of Afrikaans/Xhosa.

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- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) .....…..…….. on (*date*) ……...…….. 2012.

.....

Signature of interpreter Signature of witness

APPENDIX C: PHYSICAL ACTIVITY DIARY

For each diary entry that you make please keep the following in mind / *Hou asb die volgende in gedagte vir elke inskrywing wat jy maak:*

Did you ?/ *Het jy?*

- Participate in any exercise training (what type? Duration? Intensity?) / *deelgeneem aan enige vorm van oefening (tipe? Tydsduur? Intensiteit?*
- Any unusual amount of walking / running/ climbing of steps as part of daily activities */ Enige buitengewone hoeveelheid loop / hardloop / trappe klim as deel van daaglikse aktiwiteit*
- Participate in any unaccustomed activities ? (not usually do) / *deelgeneem aan enige aktiwiteite wat jy nie gewoonlik doen nie?*
- Have any form of injury or bruising on your body? */ Enige beserings of bloukolle op u lyf ?*

YOU SHOULD REFRAIN FROM TAKING ANY SUPPLEMENTS (Vitamins/ immune boosters) AND ANY OTHER OVER THE COUNTER MEDICATION FOR THE DURATION OF THE STUDY – BUT IN THE UNLIKELY EVENT THAT YOU NEED TO USE MEDICATION PLEASE SPECIFY WHY AND WHAT TYPE. / *U WORD VERSOEK OM GEEN OOR DIE TOONBANK MEDIKASIE OF AANVULLINGS (Vitamiene/ immune versterkers) TE GEBRUIK TYDENS DIE STUDIE NIE, MAAR IN DIE GEVAL DAT DIT NODIG IS VIR U OM SO IETS TE GEBRUIK NOEM ASB WATTER TIPE EN WAAROM.*

Subject no. / *Proefpersoon nr*. _____ **Starting date** / *Begin datum*: __/____/_____

_____
_____
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_____

APPENDIX D: FATIGUE AND MUSCLE SORENESS QUESTIONNAIRE

Subject no. / *Proefpersoon nr*. _____ **Starting date** / *Begin datum*: ___/____/_____

Day of study / *Studie dag*: post/ 4hrs/ d1/ d2/ d3/ d4/ d7

Please answer the questions below by writing the appropriate number next to the question. / *Antwoord asb die onderstaande vrae deur die toepaslike nommer langs die vraag te skryf.*

Part 1 – fatigue/ *Deel 1 – moegheid* (**Question 1-4** */ Vrae 1-4)*

1. None/ *geen*
2. Very slight/ *baie effens*
3. Slight / *effens*
4. Mild / *geringe*
5. Moderate / *middelmatig*
6. Moderate-Severe / *middelmatig-erg*
7. Severe/ *erg*
8. Very severe / *baie erg*
9. Very very severe / *baie baie erg*
10. Totally exhausted / *total uitgeput*

1. How do you rate the fatigue you feel in your legs / *Watter graad van moegheid ervaar jy in jou bene?* _____

2. How do you rate the fatigue you feel in your hamstrings / *Watter grad van moegheid ervaar jy in jou hamstrings?* _____

3. How do you rate the fatigue you feel in your quadriceps / *Watter grad van moegheid ervaar jy in jou quadriceps?* _____

4. How do you rate the fatigue you feel in your calf muscles / *Watter grad van moegheid ervaar jy in jou kuit spiere?* _____

Part 2 – muscle soreness / *deel 2 – spier seerheid* **(Question 5-8** / *Vrae 5-8)*

1. No pain / *Geen*
2. Very slight / *Baie effens*
3. Slight / *effens*
4. Mild / *geringe*
5. Moderate / *middelmatig*
6. Moderate – severe / *middelmatig- erg*
7. Severe / *erg*
8. Very severe / *baie erg*
9. Very very severe / *baie baie erg*
10. Unbearable pain / *onuithoubaar*

5. How would you rate the pain in your Quadriceps **at rest** / *Watter graad van spier seerheid ervaar jy **tydens rus** ?*_____

6. How would rate the pain in your quadriceps **during daily activities (e.g. walking, climbing stairs, etc.)** / *Watter graad van spier seerheid ervaar jy in jou quadriceps tydens daaglikse aktiwiteite (loop, trappe klim) ?* _____

7. How would you rate the pain in your quadriceps **during a stretch (pulling your heel back towards your buttocks)?** / *Watter graad van spier seerheid ervaar jy in jou quadriceps **tydens 'n strek (trek jou hak na agter in die rigting van jou boud)?***

_____

8. How would you rate the pain in your quadriceps **when you apply pressure to them** / *Watter graad van spier seerheid ervaar jy in jou quadriceps as **jy druk daarop toepas** ?*_____

APPENDIX E: LABORATORY PROTOCOLS

E.1 Bio-Plex Pro Human Cytokine assay procedure *(Chapter 4 section 4.3.1.2)*

- The 96-well microplate was prewet by adding 100μl of assay buffer to each well and removing it by vacuum filtration.
- 50μl of already prepared 1x coupled beads were added to each well followed by two washing steps *(100ul of wash buffer followed by vacuum filtration).*
- 50μl of standards and samples were added to the respective wells and incubated on a shaking platform for 30 minutes.
- Following incubation the microplate was washed three times and 25μl of detection antibody added to each well. The microplate was once again placed on the shaking platform and incubated for 30 minutes.
- In between another two washing steps, 50μl of Streptavidin-PE was added to each well and incubated for 10 minutes.
- 125μl of Assay buffer was finally added to each well before data acquisition using the Luminex 100 system.

E.2 Enzyme-linked immunosorbent assay: Cortisol *(Chapter 4 section 4.3.1.3)*

- 20μl of each standard (with a known concentration of cortisol 0, 20, 50, 100, 200, 400 and 800 $ng.mL^{-1}$) and serum sample were dispensed into the microtiter wells. All the standards were analysed in duplicate.
- 200μl of enzyme conjugate were dispensed into each well and mixed for 10 seconds before leaving it to incubate for 60 minutes at room temperature.
- Following incubation the contents of each well was shaked out on absorbant paper and rinsed 3 times with diluted wash solution.
- 100μl of substrate solution was added to each well and incubated for 15 minutes at room temperature before adding 100μl of stop solution.
- The absorbancies were immediately read on a universal plate reader (*EL800, BioTek instruments*) at 450nm. The average absorbance values for each set of standards were

calculated and a standard curve determined by plotting the mean absorbance obtained against its known concentration.

E.3 Milliplex xMAP immunoassay procedures *(Chapter 4 section 4.3.1.4)*

E.3.1 Human MMP2 panel (#HMMP2-55K, single plex MMP-9)and human cytokine/chemokine magnetic bead panel (#HCYTOMAG-60K, 5 plex) assay procedure:

- The 96-well filter plates were prewet with 200µl of wash buffer and left to shake on the plate shaker for 10 minutes at room temperature.
- Wash buffer was removed by vacuum filtration and 25µl of each standard and control was added to the appropriate wells.
- 25µl of assay buffer was added to all the background, standard, control and sample wells before loading 25µl of each sample (diluted as per manufacturer's instructions) to the appropriate wells.
- 25µl of beads were added to all the wells and left to incubate for 2 hours at room temperature on the shaking platform.
- Following incubation, the plate was washed twice with 200µl wash buffer per well removed using vacuum filtration.
- 25µl of the specific detection antibodies were added to each well and incubated with agitation on the plate shaker for 1 hour.
- Following incubation 25µl of Streptavidin-Phycoerythrin was added to each well and incubated for 30 minutes with agitation on the plate shaker at room temperature.
- This was followed by another washing step, 200µl wash buffer per well removed using vacuum filtration.
- 100µl of sheath fluid was added to all the wells and the beads resuspended on a plate shaker for 5 minutes before running the plate on the Luminex 100.

E.4 Multicolour flow cytometric analysis *(Chapter 4 section 4.3.2.2)*

E.4.1 Circulating haematopoietic progenitor cells: Compensation experiment (Anti-mouse Ig, κ */Negative control (BSA) compensation Plus (7.5um) Particles set,BD BioSciences #560497):*

- One drop of BD CompBead Plus anti-mouse Ig *(#51-9006274)* particles which bind to any mouse κ light chain-bearing immunoglobulin and one drop of BD CompBead Negative

control (BSA) *(#51-9006227)* particles which have no binding capacity was added to 100*ul* of phosphate buffer solution (PBS).

- This was repeated four times (once for each fluorochrome). The recommended volume of each fluorochrome-conjugated mouse antibody were added to the compensation beads and incubated for 30 minutes in the dark followed by a washing step with 500*ul* of PBS.

- When analysed by flowcytometry (FACSCaliur system , *Becton Dickinson*) the compensation beads provided distinct positive and negative (background fluorescence) populations which is used in the experiment to correct for spectral overlap for the combination of fluorochrome-labelled antibodies. This correction was performed using the instrument set-up software *(BD CellQuest Pro, Becton Dickinson).*

E.4.2 Staining procedure: Circulating haematopoietic progenitor cells

- 100*µl* of whole blood (EDTA tubes) were transferred to a FACX tube and washed with 500*ul* of sterile PBS. Following the addition of PBS, the blood was centrifuged at 400 g's for 5 minutes and the supernatant discarded.
- The cells were resuspended and a cocktail of antibodies added *(20µl FITC labelled CD34 #560942, 20µl PE labelled CD 38 #560981, 20µl APC labelled CD 3 #5513783, 20µl PerCP labelled HLA-DR #347402, BD BioSciences)* . The cells were left to incubate in the dark for 15 minutes.
- Following incubation the cells was washed with 500*µl* PBS (centrifuged for 5min @ 400 g's) and the supernatant discarded. The samples were fixed by adding 500*µl* (1x) lysing solution (*BD FACS Lysing solution, #349202*) containing 4 % paraformaldehyde and analysed within 24 hours.

E.4.3 Activation status of circulating neutrophils and macrophages: Compensation experiment (Anti-mouse Ig, κ /Negative control (BSA) compensation Plus (7.5um) Particles set,BD BioSciences #560497):

- One drop of BD CompBead Plus anti-mouse Ig *(#51-9006274)* particles which bind to any mouse κ light chain-bearing immunoglobulin and one drop of BD CompBead Negative control (BSA) *(#51-9006227)* particles which have no binding capacity was added to 100*µl* of phosphate buffer solution (PBS).

- This was repeated six times (once for each fluorochrome). The recommended volume of each fluorochrome-conjugated mouse antibody were added to the compensation beads and incubated for 30 minutes in the dark followed by a washing step with 500*µl* of PBS.

- When analysed by flowcytometry (FACSCanto, *Becton Dickinson*) the compensation beads provided distinct positive and negative (background fluorescence) populations which is used in the experiment to correct for spectral overlap for the combination of fluorochrome-labelled antibodies. This correction was performed using the instrument set-up software *(BD CellQuest Pro, Becton Dickinson).*

E.4.4 Fluorochrome minus one (FMO) experiment:

- One drop of BD CompBead Plus anti-mouse Ig *(#51-9006274)* particles which bind to any mouse κ light chain-bearing immunoglobulin and one drop of BD CompBead Negative control (BSA) *(#51-9006227)* particles which have no binding capacity was added to 100*ul* of phosphate buffer solution (PBS).

- A cocktail of antibodies containing the recommended volume of each fluorochrome-conjugated antibody were added to the compensation beads. This was repeated six more times with each time a different fluorochrome-conjugated antibody missing from the cocktail of antibodies (*refer to table E.4.4*). Following the addition of the antibody cocktail, the beads were incubated for 30 minutes in the dark followed by a washing step with 500*ul* of PBS.

Table E.4.4 Fluorochrome conjugated antibody cocktails

	Tube1	Tube2	Tube3	Tube4	Tube5	Tube6	Tube7
MPO (FITC)	20µl	-	20µl	20µl	20µl	20µl	20µl
CD88 (PE)	20µl	20µl	-	20µl	20µl	20µl	20µl
CD11b (V450)	5µl	5µl	5µl	-	5µl	5µl	5µl
CD163 (APC)	5µl	5µl	5µl	5µl	-	5µl	5µl
CD68 (PerCPCy5.5)	5µl	5µl	5µl	5µl	5µl	-	5µl
CD45 (APCCy7)	5µl	5µl	5µl	5µl	5µl	5µl	-

Footnote: The recommended volume of each antibody were used per test as per manufacturer's instructions.

- When analysed by flowcytometry (FACSCanto, *Becton Dickinson*) the compensation beads provided distinct positive and negative (background fluorescence) populations which is used to correct for spectral overlap for this specific combination of fluorochrome-labelled antibodies.

E.4.5 Staining procedure: Activation status of circulating neutrophils and macrophages

- 100μl of whole blood (EDTA tubes) were transferred to a FACX tube and 1mL (1x) lysing solution *(BD FACS Lysing solution, #349202*) added and incubated for 10 minutes at room temperature.
- This was followed by a centrifugation @ 400 g's for 5 minutes. The supernatant was discarded and the cells resuspended.
- 0.5 mL Permeabilizing solution 2 *(BD FACS Permeabilizing solution 2, #340973)* was added and left to incubate for 10 minutes.
- Following incubation, the cells were washed with 500μl PBS followed by centrifugation @ 400g's for 5 minute and the supernatant discarded.
- The cells were resuspended and a cocktail of antibodies added (*refer to table 4.3.2.2 tube 1*). The cells were left to incubate in the dark for 30 minutes.
- Following incubation the cells was washed with 500μl PBS (centrifuged for 5min @ 400 g's) and the supernatant discarded.
- The samples were fixed by adding 500μl 1% paraformaldehyde solution and analysed within 24 hours.

E.5 Peripheral Blood Mononuclear Cell isolation procedure: *(Chapter 4 section 4.3.3)*

- 3mL Histopaque-1077 (*Sigma-Aldrich,#RNBB9926*) were added to a 15ml falcon tube with an equal volume (3mL) of whole blood carefully layered onto the histopaque-1077. This was followed by centrifugation at room temperature @ 400 g's for 30 minutes.
- The upper layer of plasma was discarded and the opaque interface containing the PBMC's were transferred to a clean 15mL falcon tube.
- 10mL sterile PBS were added and centrifuged at room temperature @ 250 g's for 10 minutes.
- The supernatant was discarded and the cells resuspended with 5mL sterile PBS, followed by centrifugation at room temperature @ 250 g's for 10 minutes.
- The supernatant was once again discarded and the cells resuspended and frozen @ -80 °C in a 20% human serum albumin (HSA) (*Low-endotoxin, Sigma-Aldrich,#A5843-5G*) and 20% dimethyl sulfoxide (DMSO) solution.

E.5.1 Nuclear vs cytoplasmic localization of Nuclear Factor kappa β p65: Immunofluorescent staining procedure.

- 100µl of PBMC's resuspended in PBS were added per well of a 8-well staining chamber and left to adhere for approximately 15 minutes.
- 100 µl of fixative (methanol) were added per well and incubated for 10 minutes @ 4°C.
- Following incubation the cells were washed twice with 100 µl sterile PBS and 100 µl Triton X-100 (*MERCK, #1014224*) added per well and incubated for 10 minutes @ 4°C.
- Following another washing step (2x 100 µl PBS), 100 µl 5% goat serum was added to each well and incubated for 20 minutes at room temperature.
- The serum was drained and 100 µl of [1:100] primary antibody (*Rabbit polyclonal NFκB p65, abcam, #ab16502*) added to each well and left to incubate for 90 minutes at room temperature.
- Following incubation, the cells were washed twice with 100µl sterile PBS and 100 µl of [1:250] secondary antibody (*Alexa Fluor 594, goat anti-rabbit, Invitrogen*) were added and left to incubate for 30 minutes in the dark at room temperature.
- Following another washing step, 100 µl of [1:10 000] Hoescht (*bisBenzimide H33342 trihydrochloride, 099K400, #B2261-100MG*) were added per well and incubated for 10 minutes in the dark at room temperature.
- Following incubation cells were washed with 100 µl PBS and frozen @ -20°C until analysis at a later stage.

E.6 Western blotting and SDS-page *(Chapter 4 section 4.3.4)*

E.6.1 Bradford protein quantification

- For each biopsy 5µl of lysate, 95µl of distilled H_2O and 900µl Bradford working solution *(Bradford stock: 500 mg Coomassie Brilliant blue G, 250 ml 95% ethanol, 500 ml phosphoric acid, up to 1 L with dH_2O. Bradford working solution: bradford stock diluted in 1 to 5 ratio with dH_2O and filtered twice)* were prepared together with a standard curve containing known amounts of protein (0, 2, 4, 8, 12, 16, 20 *ug*) using 1mg.ml^{-1} Albumin from bovine serum (BSA).

- The absorbancies of the standard curve and each sample were read at 595 nm wavelength using a spectrophotometer *(Cary 50 Conc UV-visible*). This data was then used to determine the protein concentration in each lysate.

E.6.2 Western blotting procedure

1.0 mm Acrylamide gels containing 10 wells were used in combination with the BioRad western blotting system for separating the proteins in the extracted samples and were prepared as follow:

- 10% separating gel *(7700*µl *dH_2O, 5000*µl *1.5 M Tris-HCl pH 8.8, 200*µl *10% SDS, 40*µl *10% APS (0.1g/ml), 5000*µl *40% Acrylamide, 10*µl *Temed)* were prepared and allowed to set for 1 hour.

- After the separating gel was set, the 4% stacking gel *(6100* µl *dH_2O, 2500* µl *1.5M Tris-HCl pH 8.8, 100* µl *10% SDS, 100* µl *10% APS (0.1g/ml), 1000* µl *40% Acrylamide, 10* µl *Temed)* was prepared and allowed to set for 30 minutes.

- Running buffer *(60.6 g Trizma base, 288 g Glycine, 20 g SDS, up to 2 L with dH_2O, diluted 1:10 before use*) was added to the outer chambers of the BioRad system and the electrodes attached. An initial 10 minute run was performed at 100 V (constant) and 400 mA, thereafter a longer run of about 50 minutes at 200 V (constant) were performed.

- After this an electro transfer from the gels onto PVDF membranes *(Immobilon Transfer membrane, Millipore, # IPVH 00010)* were performed on a semi-dry transfer cell *(Bio-Rad Transblot SD)* using thick BioRad blocking paper and ready to use Tris/Glycine buffer *(BioRad, #161-0771)*. The transfer was performed at limit 1.5 A and 15 V for one hour. Before and after the transfer the PVDF membranes were placed in methanol for 15 seconds in order to activate the membranes and to keep the proteins bound.

- After the transfer was completed the membranes were washed in copious amounts of (TBS-T) 3 changes, 5 minutes each and then blocked in 5% non-fat milk *(5g/100ml TBS-T)* for 30 minutes on the shaking platform *(Stoval life science Inc., belly dancer # 4702610)*.

- After blocking, the membranes were placed on the primary antibody (*refer to table 4.3.4*) in the wall freezer @ 1.5 °C overnight.

***** The next day the membranes were washed with TBS-T before they were placed on the HRP-conjugated secondary antibody (*refer to table 4.3.4*) and incubated for one hour with agitation. Membranes were washed again in TBS-T and exposed on film in the dark room using an ECL detection kit *(GE healthcare, Amersham RPN2108)*.

- After developing; the membranes were completely stripped of all antibodies *(in dH_20, 0.2M NaOH, dH_2O)*. This was followed with blocking in 5% non-fat milk for 30 minutes before placing the membranes on a primary antibody against β-actin *(Santa Cruz biotechnology, sc-81178)* overnight. The next day the process (indicated by *****) was repeated and membranes exposed on film.

E.7 Phospho protein detection assay procedure*: (Chapter 4 section 4.3.5)*

- The 96-well microplate was washed *(100*µl *wash buffer per well followed by vacuum filtration)* .
- 50 µl of prepared (x1) coupled beads *(85*µl *IkBα specific coupled beads and 85*µl *NFκB p65 (x50) specific coupled beads , 4080*µl *wash buffer)* were added to each well followed by immediate vacuum filtration.
- The 96-well microplate was then washed *(100*µl *wash buffer per well followed by vacuum filtration*) twice.
- 50µl of the control lysates as well as the experimental lysates were added to the respective wells and incubated overnight on a shaking platform.
- The next day following incubation, the microplate was washed *(100*µl *wash buffer per well followed by vacuum filtration)* three times before adding 25µl of the specific detection antibodies *(85*µl *IkBα antibody and 85*µl *NFκB p65 antibody, 1995*µl *detection antibody diluent)* and leaving it to incubate for 30 minutes.
- This was followed by vacuum filtration and a single washing step washed *(100*µl *wash buffer per well followed by vacuum filtration)*.
- 50µl Streptavidin-PE *(42.5*µl *Streptavidin PE, 4207.5* l *wash buffer)* was then added to each well and incubated for 10 minutes.

The microplate was vacuum filtered and rinsed (*100*µl *resuspension buffer per well followed by vacuum filtration)* before adding 125µl of resuspension buffer to each well .

E.8 Myosin ATPase Staining protocol: *(Chapter 4 section 4.3.7)*

(modified from Gollnick et al. 1983)

- The slides with the cryostat sections were pre-incubated in 0.2M Acetate buffer pH 4.3 for 15 minutes. *(0.2 M Acetate buffer pH 4.3 – 40ml 0.2 M acetic acid, 20ml 0.2 M sodium acetate. pH levels were adjusted using 0.1M HCl or 0.1M NaOH).*

- Slides were rinsed in pH 9.4 buffer and then incubated for 5 minutes in the pH 9.4 buffer. *(Veronal Buffer pH 9.4 – 50ml 0.1 M sodium barbitone, 50ml 0.18 M $CaCl_2$, 150ml distilled H_2O)*

- This was followed with incubation in a pre-warmed substrate solution at 37°C in a shaking water bath for 24 minutes. *(Substrate solution – 10 ml 0.1 M sodium barbitone, 5ml 0.18 M $CaCl_2$, 35ml distilled H_2O, 150mg ATP, solution was left to warm before adjusting pH to 9.4 with either 0.1M HCl or 0.1M NaOH).*

- After incubation in substrate solution all sections were soaked in 0.09 M $CaCl_2$ for 3 minutes and placed in 2% cobalt chloride for a further 3 minutes.

- Sections were then washed in 0.01 M sodium barbitone and rinsed in distilled water before collecting them in a slide rack and developing colour in 1% ammonium sulphide for 30 seconds.

- After the colour was developed the slides were washed in distilled water, mounted and stored at room temperature.

E.9 Immunohistochemistry staining protocol: *(Chapter 4 section 4.3.8)*

- After washing slides in PBS (3 changes, 5 min each). *(PBS – 2 g KCl, 80 g NaCl, 11.6 g Na_2HPO_4, 2 g KH_2PO_4, dH_2O up to 10 L, pH 7),* the sections were dried and marked around with a wax pen.

- Thereafter sections were incubated with 20% goat blocking serum (*5 ml PBS w/ 2% BSA (0.1 g), 0.2 % milk powder (0.01 g) * 500 ul solution with 100 ul goat blocking serum*) for 30 min at room temperature.

- This was followed with overnight incubation at 4°C with the first primary antibody (*mouse monoclonal*).

- The next day sections were washed with PBS (3 changes, 5 min each) before administering the secondary antibody *(goat anti-mouse IgG*) diluted to [1:200], in the dark for 60 min at room temperature.
- This was followed by another washing step with PBS (3 changes, 5 min each). Slides were then incubated with Laminin *(Polyclonal Rabbit)* diluted to [1:250], in the dark for 4 hours.
- Sections were washed again with PBS (3 changes, 5min each) and incubated with the next secondary antibody (*goat anti-rabbit*) diluted to [1:200] in the dark for 60 min @ room temperature.
- Another washing step with PBS (3 changes, 5 min each) was followed by incubation with Hoechst diluted [1:200], for 15 min in the dark.
- All sections were mounted using fluorescent mounting medium *(DAKO, North America, USA #10030594)* and stored @ –20°C .