Satellite Cell Proliferation in Response to a Chronic Laboratory-Controlled Uphill vs. Downhill Interval Training Intervention

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously, in its entirety or in part, submitted it at any university for a degree

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Signature .....

Date .....

ABSTRACT

Despite a growing interest into the mechanisms of the repeated bout effect, little is known about the consequences of chronic eccentrically biased training and the possible advantageous such training may offer to athletes as well as patients with muscle-debilitating disease. This study investigated the role of satellite cells in the muscle adaptation in response to either downhill or uphill high intensity training (HIT). Well-trained endurance runners were divided into two training groups matched for training volume and 10 km running times (n = 6, uphill training, UP; n = 6, downhill training, DH) and subjects in both groups completed 10 HIT sessions over a period of 4 weeks. Running performance was tested before and after the training intervention by a 10 km road race and peak treadmill speed (PTS) in horizontal and inclined (+5%) laboratory incremental tests to exhaustion. Skeletal muscle biopsies were sampled at baseline, after 2 HIT sessions, and after 4 weeks of HIT. Muscle was analysed immunohistochemically for satellite cell frequency as identified by CD56 and M-cadherin (Mcad) expression. Myogenin protein contents of muscle homogenates were determined by western blotting. Myosin heavy chain (MyHC) isoform proportions and mean fibre cross-sectional area was measured. During the HIT intervention, UP exercised at a higher percentage of their HR_{max} than DH (mean ± SD, 97 ± 1 vs. 92 ± 3 $\%HR_{max}$, $p < 0.005$), but at a similar rate of perceived exertion (RPE). DH completed more intervals per session and covered greater distance per session than their UP counterparts. Both training groups increased their training intensity but decreased their training volume during the 4 weeks of HIT. The combined group of 12 athletes improved their $PTS_{gradient}$ (mean ± SD, 16.7 ± 0.8 vs. 17.3 ± 1.0 km/h, $p < 0.05$). No significant differences between groups were found for PTS, VO_2max or 10 km performance. Satellite cell frequency in this cohort of trained runners (48.9 ± 10.3 km/week) at baseline was similar to healthy young males ($CD56^+$ cells/fibre, 0.19 ± 0.10). Satellite cell frequency increased significantly in DH after 4 weeks (Mcad, 123%; CD56, 138%) and non-significantly in UP (Mcad, 45%, CD56, 39%). No significant differences were found after two training sessions or at any time between groups. Mcad and CD56 expression correlated well ($r = 0.95$, $p < 0.0001$). Muscle myogenin content increased for both groups (UP: 56%; DH: 60%) after 4 weeks. No notable changes were seen after two training sessions. However, myogenin levels 2 days after session 1 correlated well ($r= 0.99$, $p<0.005$) with muscle pain experienced on the same day, as measured by the visual analogue scale. No changes were seen in the MyHC proportions or the fibre cross-sectional area after the training intervention. It was concluded that the training intervention was too short to induce changes in MyHC distribution or fibre area. Is seems likely that satellite cell proliferation was initiated as an early response to DOMS, but the response was maintained for 4 weeks. However, due to the lack of change in fibre morphology and myonuclear number, the role of satellite cell proliferation in fibre type transformation or muscle hypertrophy could not be established. Similarly, various possible roles for increased myogenin protein are offered, but since the origin of myogenin expression (satellite cells vs. myonuclei) was not determined, no definite conclusion regarding the precise function can be made. In conclusion, this study is the first to definitively indicate satellite cell proliferation in well-trained endurance runners in response to a change in training, including specifically downhill HIT. This response was early and sustained. This study asks several questions about the role of satellite cells during muscle adaptation to repetitive downhill training, and lays a foundation for further research into this unexplored field.

OPSOMMING

Ten spyte van die groeiende belangstelling in die onderliggende meganismes van die "herhaalde beurt effek" is daar min kennis oor die gevolge van kroniese essentriese oefening en die moontlike voordele wat sulke oefening vir atlete asook patiënte met spier-verswakkende siektes mag inhou. Hierdie studie het die rol van satellite selle in spier adaptasie in reaksie op óf afdraende óf opdraende hoë-intensiteit oefening (HIO) ondersoek. Goed-geoefende langafstand atlete is opgedeel in twee oefengroepe gepaar vir oefeningvolume en 10 km hardloop tye (n = 6, opdraende oefening, UP; n = 6, afdraende oefening, DH) en proefpersone in beide groepe het 10 hoë-intensitiet oefenings-sessies oor 'n tydperk van 4 weke voltooi. Hardloop presatsie is getoets voor en na die oefeningsintervensie deur 'n 10 km padwedloop en top trapmeul spoed (TTS) tydens horisontale en opdraende (5%) laboratorium toenemende-toetse tot vermoeienis. Skeletspier biopsies is geneem voor basislyn toetse, na twee HIO sessies, en na 4 weke van HIO. Spier is immunohiostologies geanaliseer vir satelliet sel frekwensie soos geidentifiseer deur CD56 en M-cadherin uitdukking. Myogenin spier hoeveelheid van spier is bepaal deur immunoblotting. Myosin swaar ketting (MyHC) isoform proporsies en gemiddelde vesel dwarssnee area is bepaal. Gedurende die HIO intervensie het UP geoefen teen 'n hoër persentasie van hul maksimum hartklop as DH (mean ± SD, 97 ± 1 vs. 92 ± 3 $\%HR_{maks,}$ $p < 0.005$), maar teen 'n soortgelyke syfer van waargenome inspanning. DH het meer intervalle per sessie en 'n groter afstand per sessie voltooi as UP. Beide oefenings groepe het hulle oefenings-intensiteit verhoog maar hulle oefenings-volume verlaag gedurende die 4 weke van HIO. Die gekombineerde groep van 12 atlete het hul $TTS_{opdraend}$ verhoog (mean ± SD, 16.7 ± 0.8 vs. 17.3 ± 1.0 km/h, $p < 0.05$). Geen beduidende verskille is tussen groepe is gevind vir TTS, $V0_{2maks}$ of 10 km presatsie nie. Satelliet sel frekwensie in hierdie groep geoefende atlete by basislyn (48.9 ± 10.3 km/week) was soortgelyk aan dié van gesonde jong mans ($CD56^+$ cells/fibre, 0.19 ± 0.10). Satelliet sel frekwensie het beduidend gestyg in DH na 4 weke (Mcad, 123%; CD56, 138%) en nie-beduidend in UP (Mcad, 45%, CD56, 39%). Geen beduiende verskille is na twee oefenings-sessies in enige van die groepe of tussen die groepe gevind nie. M-cadherin en CD56 uitdrukking het goed gekorreleer ($r = 0.95$, $p < 0.0001$). Spier myogenin hoeveelheid het vermeerder in beide groepe na 4 weke (UP: 56%; DH: 60%). Geen noemingswaardiege verskille is na twee oefenings sessies gesien nie. Myogenin vlakke 2 dae na die eerste HIO sessie het goed gekorreleer met spierpyn ervaar op dieselfde dag ($r= 0.99$, $p<0.005$), soos gemeet deur die visuele analoog skaal. Geen verskille is gesien in MyHC proporsies of vesel dwarssnee area na die oefenings-intervensie nie. Dit is beslis dat die oefenigs-intervensie van te korte duur was om veranderinge in MyHC verspreiding of vesel area te veroorsaak. Dit blyk moontlik dat satelliet sel proliferasie geïnisieer is as 'n vroeë respons tot 'vertraagde aanvang spier-seerheid', maar die repons is gehandhaaf vir 4 weke. As gevolg van die tekort in verandering in vesel morfologie en spier-nukleus aantal kon die rol van satelliet sel proliferasie in vesel tipe veranderinge of spier hipertrofie nie vasgestel word nie. Op dieselfde manier word verskeie moontlike rolle vir verhoogde myogenin proteïn gegee, maar aangesien die oorsprong van myogenin uitdrukking (satelliet selle teenoor spier-nukleusse) nie bekend is nie, kon geen defnitiewe slotsom oor die funksie gemaak word nie. Ten slotte, hierdie studie is die eerste om aan te dui dat satelliet sel proliferasie in geod-geoefende langafstand atlete voorkom in reaksie op 'n verandering in oefening, insluitend spesifiek afdraende HIO. Hierdie respons was vroeg asook volgehou. Hierdie studie vra verskeie vrae oor die rol van satelliet selle gedurende spier adaptasie ingevolge afdraende oefening, en dit lê 'n fondasie vir verdure navorsing in dié onbekende veld.

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ABBREVIATIONS

ANOVA	analysis of variance	***n***	number of subjects
ATP	adenosine triphosphate	**Na_2HPO_4**	sodium hydrogen phosphate
BSA	bovine serum albumin	**NaCl**	sodium chloride
CO_2	carbon dioxide	**NaF**	sodium fluoride
Con	concentric	**N-CAM**	neural cell adhesion molecule
CSA	cross-sectional area	**Nm**	Newton meter
dH_2O	distilled water	**NP40**	nonidet P40
DOMS	delayed onset muscle soreness	**NS**	not-significant
DTT	dithiothreitol	**p**	probability
Ecc	eccentric	**PAGE**	polyacrylamide gel electrophoresis
EDTA	ethylene diamine tetra-acetic acid	**PBS**	phosphate buffered saline
EGTA	acetic acid	**PCNA**	proliferating cell nuclear antigen
FGF	fibroblast growth factor	**PMSF**	Phenylmethylsulphonylfluoride
HCl	hydrochloric acid	**PTS**	peak treadmill speed
HGF	hepatocyte growth factor	**PVDF**	polivinylidene fluoride
HIT	high intensity training	**r**	Pearson's correlation coefficient
HR	heart rate	**reps**	repetitions
IGF-1	insulin-like growth factor 1	**RM**	repetition maximum
IL-6	interleukin 6	**RPE**	rate of perceived exertion
KCl	potassium chloride	**RT**	room temperature
kDA	kilo Dalton	**SBTI**	soybean trypsin inhibitor
KH_2PO_4	potassium dihydrogen phosphate	**SD**	standard deviation
leu19	leukocyte differentiation antigen 19	**SDS**	sodium dodecyl sulphate
LIF	leukaemia inhibiting factor	**SE**	standard error of the mean
M	Molar	**TBS**	tris buffered saline
mATPase	myofibrillar adenosine triphosphatase	**TGF**	transforming growth factor
M-cadherin	muscle cadherin	**TT**	time trial
MGF	mechano growth factor	**V**	Volt
MNF	myocyte nuclear factor	**v/v**	volume per volume
MRF	myogenic regulatory factor	**VAS**	visual analogue scale
mRNA	messenger ribonucleic acid	**VO_2**	oxygen consumption
MVC	maximum voluntary contraction	**W**	Watt
MyHC	myosin heavy chain	**w/v**	weight per volume

CHAPTER 1: INTRODUCTION

Skeletal muscle is one of the most responsive and adaptable tissues in the human body. It responds to different stimuli in a dynamic manner, changing in quantity, quality and internal environment within short time constraints. This is clearly illustrated by the muscular adaptations of athletes from different sporting disciplines, and in stark contrast, those of people with debilitating muscular diseases and muscle atrophy. The dynamic nature of muscle adaptation has made it a popular tissue to investigate physiological processes such as regeneration, growth, and cellular energy metabolism.

The stimuli for and the mechanisms of skeletal muscle adaptation have specific relevance to athletic performance. Athletes continuously seek training methods to more effectively produce the desired adaptation e.g. changes in speed and power. However, exercise physiologists seek to understand the underlying mechanisms of adaptation. Currently much research focuses on molecular and cellular changes within muscle, ranging from metabolic enzymes' activities and modulation of their gene expression to e.g. presence of cell surface or membrane proteins and associated signal transduction pathways.

Equally important are the mechanisms of muscle adaptation during muscular diseases such as muscular dystrophies and muscle atrophy associated with HIV, cancer and aging. Central to prevention of muscular atrophy, as well as advances in athletic performance, are the mechanisms underlying increases in muscle strength and size (hypertrophy). A very relevant experimental model to examine these mechanisms is to study the adaptations brought about by a change in exercise routine. This model can also be directly applicable if the intervention affects whole body physical capacity and athletic performance and can easily be applied to the development of effective training or treatment strategies.

To understand the responses to exercise training, much attention has been given to the mode of muscle contraction. It seems eccentric muscle contraction, as a stimulus for adaptation, may function differently from concentric muscle contraction, and may even provide additional benefits.

The major theme of the following literature review is to discuss the training adaptations to eccentric exercise. Often this is clearly illustrated through direct comparison with concentric exercise, as the opposing mode to eccentric exercise. Understanding the primary muscle damage inflicted by one bout of eccentric contraction is fundamental to understanding adaptations to eccentric exercise. However, the early biomechanical and biochemical responses to eccentric contraction will be mentioned only briefly, as they are not the focus of this thesis.

Rather, I will focus on previous studies that have investigated repetitive (multiple sessions of) eccentric exercise. Particular emphasis will be given to mechanisms of muscle regeneration and shifts in muscle quantity and quality, for example the changes that occur in fibre type due to exercise. As one of the central themes in this thesis is the role of satellite cells in muscle adaptation, I will provide a detailed review on the role of muscle stem cells (satellite cells) in repair and adaptation to exercise. I will also discuss selected growth factors and other regulatory factors that are relevant to muscle adaptation, and specifically to satellite cell regulation, in the context of this thesis.

Muscle adaptation has been extensively researched in cell culture and animal models. I will occasionally refer to this literature to establish key principals, but for most of the literature review, I will concentrate on studies in human subjects, especially when reviewing training adaptations and responses to exercise.

Thereafter, the thesis will describe an original experiment consisting of 4-weeks of either an uphill or a downhill high intensity training intervention in already training endurance runners. In addition to performance change, the mechanisms of muscle adaptation investigated included changes in muscle morphometry, satellite cell frequency and the myogenic regulatory factor, myogenin. Cellular markers chosen were CD56 and m-cadherin, both used for the immunohistochemical identification of quiescent and activated satellite cells.

CHAPTER 2: LITERATURE REVIEW

2.1 ECCENTRIC EXERCISE

An eccentric contraction occurs when the muscle fibres are lengthened or stretched while contracting. This happens when the external tension applied to a muscle exceeds the tension that would be developed at constant muscle length (Lindstedt *et al.*, 2001). This is in contrast to a 'typical' concentric contraction where the muscle shortens during contraction. Compared to isometric (constant length) and isotonic (shortening against a constant load) muscle contractions, eccentric contractions are characterized by high force production (Lindstedt *et al.*, 2001).

Records of scientific study into eccentric exercise date back to the early 1950's when Abbot, Bigland and Ritchie showed that eccentric exercise requires less energy expenditure than an equal volume of concentric exercise (see section 2.1.6) (Abbott *et al.*, 1952). During the next three decades research was limited mainly to the investigation of the energy dynamics and metabolism of eccentric and concentric exercise. Then, in 1981, Friden *et al.* (1981) were first to show that muscle is morphologically altered by eccentric exercise. Their discovery of the misalignment of sarcomeres after exercise initiated a shift in research focus towards the intramuscular level. While the amount of research about eccentric exercise has been ever increasing since these early years, the underlying causes of intra-muscular changes are still a much-debated issue (see section 2.1.1). Only with further research will the molecular behaviour of the contractile proteins and connective tissue themselves during eccentric contraction become clearer. Despite uncertainties regarding immediate intramuscular changes during eccentric exercise, in recent years much research has been done to investigate the long term consequences of repeated eccentric exercise.

2.1.1 Eccentric exercise-induced muscle damage and 'training effects'

The most renowned characteristic of eccentric exercise is the severe muscle pain and stiffness that peaks 2 days after an unaccustomed eccentric exercise bout; appropriately termed *delayed onset muscle soreness* (DOMS). DOMS is characterized by passive muscle pain, decreased force production, increased serum creatine kinase activity, structural disruption of sarcomeres, inflammation and increased proteolytic enzyme activity (Cleak & Eston, 1992;Friden *et al.*, 1983b;Gibala *et al.*, 1995;Malm *et al.*, 2004;Newham *et al.*, 1986;Stupka *et al.*, 2000).

A second distinctive characteristic of eccentric-induced muscle damage is the muscle adaptation that it provokes. After a bout of pain-inflicting eccentric exercise, a person will experience drastically less pain and stiffness from another similar eccentric exercise bout (Byrnes *et al.*, 1985;Newham *et al.*, 1987). The first bout of eccentric exercise provides a 'protective' effect against further damage of a similar nature. The protective effect is present within 24 hours after an eccentric bout (Chen & Hsieh, 2001) and has been shown to last for up to 6 months (Nosaka *et al.*, 2001). This is called the 'repeated bout effect'. Even when a second eccentric bout occurs before full recovery from the first bout, the damage is not exacerbated and it does not slow down the recovery rate (Ebbeling & Clarkson, 1990). The same has been shown when 3 eccentric bouts are performed consecutively within 9 days (Nosaka & Clarkson, 1995;Sorichter *et al.*, 1997). Even with 7 days of consecutive eccentric exercise, only the initial exercise bout inflicts significant muscle damage (Chen & Hsieh, 2001). Friden *et al.* have shown that the adverse effects from eccentric contractions can be totally eliminated when eccentric exercise is continued chronically over several weeks (Friden *et al.*, 1983a;Friden, 1984).

2.1.2 Underlying mechanisms of eccentric muscle damage and adaptation

The underlying causes of eccentric muscle damage are extremely complex and the field is filled with conflicting theories. However, closely associated with these theories are the

mechanisms responsible for the protective adaptations against damage from recurrent eccentric exercise. Several hypotheses have been presented to explain the repeated bout or rapid training effect. Since these are not the focus of this thesis they will not be reviewed here in great detail but the reader is referred to selected reviews on the topic which illustrate the changing understanding of the fairly acute events (McHugh, 2003;Morgan & Allen, 1999). In short, the theories developed to explain the repeated bout effect can be divided into several broad categories: neural, mechanical, cellular adaptations, adaptations in excitation-contraction coupling and changes in the inflammatory response.

Neural adaptations

This theory predicts that the initial insult is a result of high stress on a relatively small number of fast twitch fibres (Moritani *et al.*, 1987). During subsequent eccentric exercise, the neuromuscular system adapts by increasing the recruitment of larger motor units and slow-twitch motor units. These changes distribute the contractile stress over a larger number of active fibres (Nosaka & Clarkson, 1995). An increase in EMG amplitude (Hortobagyi *et al.*, 1996a) and a decrease in frequency (Warren *et al.*, 2000) seems to confirm increased fibre recruitment and increased recruitment of slow-twitch fibres respectively. However, the repeated bout effect can occur independently of neural adaptations, as demonstrated by a model using investigator-controlled neuromuscular recruitment through electrically stimulated eccentric contractions (Nosaka *et al.*, 2002).

Mechanical theory of muscle damage

The mechanical theory focuses on damage that alters the musculoskeletal system on a whole muscle level as well as on a myofibrillar level. This theory hypothesizes that muscle damage occurs when the non-contractile connective tissue elements and intermediate filaments are disrupted and myofibrillar integrity is lost (McHugh, 2003). Increased active and passive muscle stiffness provide direct and indirect evidence of

adaptations in intramuscular connective tissue (Lapier *et al.*, 1995) and cytoskeletal proteins (Pousson *et al.*, 1990). However, there is also evidence that seems to be in contrast, e.g. subjects with stiffer muscles at baseline are prone to more severe eccentric muscle damage than subjects with compliant muscles (McHugh *et al.*, 1999). Also, while increases in desmin after eccentric damage seem to reflect a mechanism for mechanical reinforcement (Barash *et al.*, 2002;Lieber *et al.*, 1996), desmin-lacking mice show less myofibrillar disruption after eccentric exercise (Sam *et al.*, 2000). These apparently conflicting lines of evidence remain to be integrated into a more plausible theory.

Cellular adaptations

This theory predicts that muscle damage is the result of irreversible sarcomere strain during eccentric contractions (Morgan, 1990). Sarcomeres are stretched non-uniformly, with some (but not all) sarcomeres stretched beyond myofilament overlap, causing a loss of contractile integrity (Friden *et al.*, 1981;McHugh *et al.*, 1999). Muscles should therefore adapt through increasing the amount of sarcomeres in series and thereby reducing sarcomere strain during a repeated bout of eccentric exercise (Morgan, 1990). Addition of sarcomeres has been confirmed in animal models (Lynn *et al.*, 1998;Lynn & Morgan, 1994) and in human subjects, where a rightward shift in the length-tension curve following an initial eccentric bout indirectly suggests a lengthening of the muscle (Brockett *et al.*, 2001).

Although it has been shown that sarcomeres in series were added in response to chronic submaximal eccentric loading consisting of downhill running (Lynn *et al.*, 1998), other authors showed that submaximal eccentric weight-training does not offer protection against subsequent maximal contractions (Nosaka & Newton, 2002). Further evidence suggesting that this adaptation does not always occur includes the finding of the temporary nature of the length-tension shift (Jones *et al.*, 1997).

Adaptations in excitation-contraction coupling

Some authors do not promote the belief that decreased performance associated with DOMS is due to muscle damage, but rather that a failure to activate intact force-generating structures within the muscle fibre is responsible for the early strength loss observed after eccentric exercise (McHugh, 2003;Warren *et al.*, 2001). Adaptations in excitation-contraction coupling may explain reduced strength loss and reduced DOMS following a repeated bout of eccentric exercise, possibly through 'strengthening' of the sarcoplastic reticulum structures (Clarkson & Tremblay, 1988). However, strength loss is similar immediately after successive bouts and only differs in capacity to recover days afterwards. This brings up two important points. Firstly, excitation-contraction coupling changes may be only one in a series of adaptive events (Balnave & Thompson, 1993;Clarkson & Tremblay, 1988). Secondly, not enough studies have been investigating mechanisms involved in the process of recovery.

Inflammatory adaptations

After eccentric contraction induced injury, the primary disruption of myofibrils elicits a local inflammatory response which can aggravate damage (secondary damage) before recovery ensues (Pizza *et al.*, 1996). Reduced damage following a repeated bout may be due to a blunted inflammatory response leading to decreased secondary myofibril disruption (Pizza *et al.*, 2002). However, an alternative explanation is that the reduced inflammatory response to a repeated bout may mirror reduced primary mechanical damage, and hence less of a stimulus for inflammation (McHugh, 2002).

It is doubtful that one of these theories can explain all the observations made about the repeated bout effect. The reality is most likely a combination of neural, mechanical, cellular and biochemical mechanisms and may depend on the specifics of the exercise protocol followed.

2.1.3 Eccentric vs. concentric resistance training

Eccentric contractions are often purposefully included in exercise routines by weightlifters and bodybuilders to produce superior gains in strength and muscle size (hypertrophy). Research indicates that eccentric contractions result in greater force production than concentric contractions (Fitzgerald *et al.*, 1991;Hortobagyi *et al.*, 1996b;Hortobagyi *et al.*, 1996a;Komi & Buskirk, 1972). Strength gains may be a function of the magnitude of force production, irrespective of the type of contraction (Friedmann *et al.*, 2004;Hortobagyi *et al.*, 1996b;Hortobagyi *et al.*, 1996a;Hortobagyi *et al.*, 2000). Thus greater maximal force production during eccentric contractions would result in greater gains in strength and muscle size, unless the load of concentric training was adapted. In contrast, a greater hypertrophy effect has been shown in response to concentric training when equal power was used (Mayhew *et al.*, 1995). But no difference in strength and hypertrophy between concentric and eccentric training has been observed (Johnson *et al.*, 1976;Jones & Rutherford, 1987;Smith & Rutherford, 1995).

To delineate differences that might explain these contrasting findings, the study designs, protocols and equipment used are summarized in Table 2.1. Examining these studies critically, it is apparent that eccentric training causes the greatest improvement in eccentric strength and likewise concentric training causes the greatest improvement in concentric strength. Greater strength increases following eccentric training is likely the result of greater force production, since they are consistently seen when maximal effort training was done. Although strength adaptations seem to be specific to type of training, muscle fibre hypertrophy may be influenced by the force of the contraction as well as the type of contraction, as greater changes in hypertrophy are seen during eccentric training than during concentric training, even when the force of contraction is similar.

Table 2.1 ***Summary of literature comparing eccentric and concentric strength training***

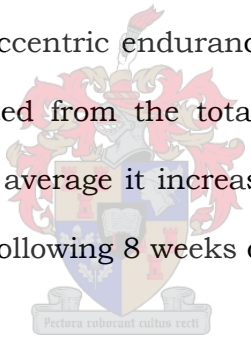
Study	Subject number & gender	Muscles trained	Matching	Training intensity	Training duration & frequency	Eccentric strength	Concentric strength	Isometric strength	Muscle size/ fibre area	Conclusion
Hortobagyi et al, 1996a	21M	*quadriceps*	random	Maximal effort	4-6 sets x 8-12 reps 3 x week, 12 weeks				(type II fibres)	Adaptation specific to
Eccentric	7				1890 reps	+ 116% ✱ ✦	+ 5% ✱	+ 45% ✱	+ 37% ✱ ✝	contraction
Concentric	8				1890 reps	+ 10% ✱	+ 53% ✱	+ 36% ✱	+ 3.1%	type
Hortobagyi et al, 1996b	42 F	*quadriceps*	Strength matched	Equal force	4 sets x 6-10 reps 4x week; 6 weeks					Ecc training increased ecc &
Eccentric group	14			Sub-max	824 reps	+ 42% ✱ ✦	+ 14%	+ 30% ✱ ✝		Isometric strength
Concentric group	14			max	824 reps	+ 13%	+ 36% ✱	+ 18% ✱		more than con
Hortobagyi et al, 2000	48			Maximal effort	4-6 sets x 8-12 reps 12 weeks, 1866 reps				(type I, IIa, IIx)	Eccentric training caused
Eccentric	6M & 6F	*quadriceps*	random			+ 86% ✱	+ 25%✱	+ 52% ✱ ✝	+10, 16, 16%✱ ✝	greater changes
Concentric	6M & 6F					+ 20%	+ 44% ✱	+ 31% ✱	+4, 5, 5% ✱	in strength and
Ecc/Con	6M & 6F					+ 70% ✱	+ 40% ✱	+ 46% ✱ ✝	+11, 9, 10% ✱ ✝	muscle fibre size
Friedmann et al, 2004	16M	*quadriceps*	random		3 x week; 4 weeks					Ecc training
Concentric/ eccentric	9			30% con 1RM	6 sets x 25 reps (45s)		No difference		No difference	caused greater
Concentric eccentric-overload	7			30% con 1RM 30% ecc 1RM	3 sets x 25 reps		+ 5% ✱		No difference	increase in strength
Mayhew et al, 1995	20	*quadriceps*	random	Equal force	5 sets x 10 reps 3 x week; 4 weeks				(type II fibres)	Isometric force and hypertrophy
Eccentric	6F & 4M			90% concentric				+ 8.0%	+ 18.0%	greater with
Concentric	8F & 2M			90% concentric				+ 16.5% ✝	+ 25.7% ✝	con training
Jones & Rutherford, 1986	5M & 1F	*quadriceps*	Intra-subject	ecc 145% > con	4 sets x 6 reps 3 x week; 12 weeks					No difference
Eccentric	6			80% ecc 1RM	↑ 261% weight			+ 11% ✱	+ 4%	
Concentric	6			80% con 1RM	↑ 250% weight			+ 15% ✱	+ 6%	
Johnson et al, 1976	8M	Arms, legs (ext + flexion)	Intra-subject		3 x week; 6 weeks					No difference
Eccentric	8			120% con 1RM	2 sets x 6 reps		Increase ✱	Increase ✱		
Concentric	8			80% con 1RM	2 sets x 10 reps		Increase ✱	Increase ✱		
Smith & Rutherford, 1995	5M & 5F	*quadriceps*	Intra-subject	10RM	4 sets x 10 reps 3 x week, 20 weeks				CSA	Greater isometric force with con
Eccentric	10			35% > con				+ 9.8% ✱	+ 4.0% ✱	training despite
Concentric	10							+ 19.6% ✱ ✝	+ 4.6% ✱	lower intensity
Komi & Buskirk, 1972	31M	forearm flexors	Isometric strength	Maximal effort	6 reps 4 x week; 7 weeks					Ecc training produces greater
Eccentric	11					+ 15.6% ✱	+ 15.8% ✱	+ 8.6% ✱ ✝	+ 1.84% ✱	gains in strength
Concentric	10					+ 6.9% ✱	+ 12.1% ✱	+ 6.3% ✱	+ 0.27%	and muscle size

✱ Significant increase from baseline ; ✝ significantly greater than same measurement from opposing training method ; ✦ Ecc strength increase in Ecc group > Con strength increase in Con group

2.1.4 Eccentric ergometry

Despite the fact that there is a magnitude of literature investigating muscle adaptation to eccentric and concentric resistance training (some of which were reviewed in the above section), studies about eccentric vs. concentric ergometry using laboratory equipment are scarce. Studies on dynamic eccentric exercise (other than resistance) are mostly limited to the investigation of delayed onset muscle soreness and the repeated bout effect, rather than assessing the training response to multiple bouts. In this section, I will review 3 eccentric training studies based in controlled laboratory settings.

In a novel study at the time, Friden *et al.* (1983a) investigated repeated eccentric training lasting 8 weeks using a specially designed cycle ergometer. Although dynamic concentric strength, assessed by maximal force, improved only slightly, an 375% improvement in maximal eccentric work capacity (eccentric endurance strength) was seen (Friden *et al.*, 1983a). This variable was calculated from the total maximal eccentric work subjects could perform until exhausted. On average it increased from 10700 Nm over 12 min at baseline to 16500 Nm over 30 min following 8 weeks of training.



More than a decade later, Lastayo *et al.* (1999, 2000) conducted similar research with the intention of provoking strength increases at eccentric exercise intensities so low that a concentric programme of equal intensity would not increase strength. Subjects cycled either eccentrically or concentrically at low training intensities for 8 weeks. Training intensity was set according to heart rate and started at 54% of HR-peak and was progressively increased to 65% HR-peak for both groups. Training frequency was increased gradually from 15 minutes two times per week to 30 minutes five times per week during the sixth week. By the end of the 8th week, the eccentric workload was nearly four times greater than the concentric workload (489 W vs. 128 W), despite both groups training at the same target HR. Only eccentrically trained subjects experienced increases in isometric leg strength (26%) and fibre cross-sectional area (52%). This may

seem unremarkable when the workloads are compared, but importantly, eccentrically trained subjects trained at the same metabolic intensity as subjects in the concentric group.

Although the studies of Friden *et al* (1983) and LaStayo *et al.* (1999, 2000) used cycle ergometry as an exercise model, their cycle ergometers really represent an alternative mode of resistance training (LaStayo *et al.*, 2000). Dynamic eccentric training with only body weight as resistance can be produced by downhill running, a mode of training also available outside of the research laboratory. But this training method is a seldom-researched area.

2.1.5 Eccentric and Concentric running

Running consists of a combination of eccentric and concentric muscle contractions (Bourdin *et al.*, 1995). Changing the slope of the running surface can change the ratio between eccentric and concentric components of this exercise. When running on a level surface, the eccentric and concentric components are equal. Only when running at gradients greater than -30% or +30% is work considered to be exclusively eccentric or concentric respectively. Running at a gradient of -10% utilizes 66% eccentric work, while running on a surface of +10% utilizes 66% concentric work (Minetti *et al.*, 1994). Thus, popular literature refers to downhill running as eccentric or negative exercise, even though this is not completely correct. The same holds for uphill running as concentric exercise.

Downhill running is often indirectly part of training routines when runners are doing hill-work and need to descend between repetitive uphill intervals. This can be compared to the eccentric work most frequently done together with concentric work during resistance training. As discussed earlier, results have shown that eccentric contractions are as important as or more important for strength development than concentric

contractions during resistance training (Hortobagyi *et al.*, 1996a;Jones & Rutherford, 1987). Does this mean that eccentric running is as important as concentric running for training adaptation? No one, to my knowledge, has studied repetitive downhill running to assess the effect of repeated eccentrically biased training on running performance and running speed.

One study (Balnave & Thompson, 1993) has investigated the effects of eccentric training using downhill walking. Results showed a decrease in muscle damage after each downhill session, assessed indirectly by creatine kinase activities and myoglobin protein levels in blood samples. Subjects completed a 40 min training session, walking at 6.4 km/h down a 25% gradient, once a week for 8 weeks and were tested each week. After the training period researchers noted a smaller reduction in maximum voluntary contraction (MVC) force immediately after exercise. Force reduction immediately after intense eccentric resistance exercise is usually unaffected by a second eccentric bout, despite improvements in muscle soreness, rested force development and less structural damage (Nosaka & Newton, 2002). The early post-exercise force improvement (lesser decline in force reductions) is consequently a function of either chronic eccentric training or the milder protocol associated with the more 'normal' exercise mode. Many adaptations to eccentric exercise that will affect performance are likely to require more than one or two sessions. After the 8 weeks of training, muscles also recovered faster from exercise-induced fatigue (Balnave & Thompson, 1993), possibly indicating that some intramuscular adaptations occurred at a metabolic level (Chin *et al.*, 1997). Balnave & Thompson (1993) concluded that their training frequency (once per week) was inadequate to produce optimal results, and that a higher training frequency might have produced greater changes.

Downhill running is often used as an easy and effective method to induce DOMS due to its predominantly eccentric nature (Baker *et al.*, 1997;Byrnes *et al.*, 1985;Feasson *et al.*,

2002;Smith *et al.*, 1998;Tsivitse *et al.*, 2003). It is widely known that muscle soreness associated with downhill exercise disappears when the event is repeated several times (Byrnes *et al.*, 1985;Chen & Hsieh, 2001;Nosaka & Clarkson, 1995). This training effect may hold several benefits for athletes, including decreased muscle fatigue and improved force development after hill training or after racing over a hilly course. Further, if the proven advantages in strength and muscle fibre size from eccentric resistance training (Friedmann *et al.*, 2004;Hortobagyi *et al.*, 2000;Komi & Buskirk, 1972) are applicable to downhill running training, it is possible that downhill running training may produce similar or greater improvements in running performance and muscle adaptation than uphill running training. This may enable athletes to run faster for extended periods or it may be reflected in improved peak treadmill speed or more specifically, enhanced hill running abilities. However, there may also be metabolic advantages over and above the structural adaptations expected.

2.1.6 Energy demands of eccentric and concentric training

One of the biggest advantages of eccentric running is the low metabolic cost involved. This was recognized as early as 1952 in an ingenious study (Abbott *et al.*, 1952). The researchers connected two stationary cycle ergometers back to back on a single chain so that one subject cycled forward and the other one resisted the forward motion by braking the backward-moving pedals. The same force was being applied by both subjects, yet the task was much easier for the subject braking. Since then, studies varying in exercise regime, have established that eccentric ergometry requires less oxygen consumption than concentric ergometry (Bigland-Ritchie & Woods, 1973;Gregor & Costill, 1973;Liefeldt *et al.*, 1992;Minetti *et al.*, 2002). Eccentric cycling at submaximal levels has even been estimated to require only 1/6 – 1/7 of the metabolic cost of concentric cycling at the same workload (Bigland-Ritchie & Woods, 1973). In the study by LaStayo *et al.* (2000) previously described, the subjects cycled eccentrically at a four-fold greater workload

than their concentrically cycling counterparts while maintaining the same exercise intensity as measured by heart rate (LaStayo *et al.*, 2000).

Liefeldt *et al.* (1992) investigated responses to horizontal and downhill running (-3° gradient) in trained runners. Oxygen consumption was less during downhill running compared to level running at an equivalent speed, while stride frequency was not statistically different and stride length was greater during downhill running. Because of the reduced metabolic costs of downhill running, athletes could achieve greater maximal speeds when running downhill compared to flat at VO_{2max} (Liefeldt *et al.*, 1992). Downhill running at fast speeds creates greater force development than during level running or uphill running, which is mostly due to the large eccentric braking component (Buczek & Cavanagh, 1990;Iversen & McMahon, 1992).

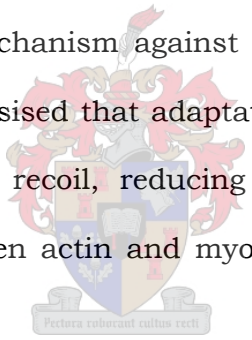
Muscle adaptation in response to resistance training, in particular strength improvement, is at least in part due to the extent of force production, irrespective of contraction type (Fitzgerald *et al.*, 1991;Hortobagyi *et al.*, 1996b;Komi & Buskirk, 1972). If the principles of resistance training can be extrapolated to high intensity training of endurance athletes, it could be that the greater force production during downhill running is a more energy efficient means of high intensity training than uphill running, with the added benefits of unique structural changes and improved fibre cross-sectional areas.

2.1.7 Eccentric training, muscle soreness and muscoskeletal injury

A major reason why downhill running is not included specifically in training routines is that it is believed to carry a greater risk of injury, including iliotibial band syndrome and over-use injuries of muscle and tendon (Clement *et al.*, 1984;Fredericson & Wolf, 2005;Gottschall & Kram, 2005;Mayfield, 1977). This reputation may be influenced to a certain extent by the muscle soreness associated with downhill running, but the other consequences are more likely due to the high braking forces needed while running

downhill. It is worth mentioning that, to my knowledge, no study has confirmed a direct relationship between downhill running and the prevalence of injuries. In contrast, eccentric resistance training has recently been implemented as a successful prevention strategy for recurring muscle tears in different sporting disciplines (Askling *et al.*, 2005;Croisier *et al.*, 2002).

Friden *et al.* (1983) obtained muscle biopsies from 9 subjects before and after 8 weeks of eccentric bicycle ergometry (2-3 times per week). Muscle biopsies were analyzed by electron microscopy for evidence of muscle damage. Significantly more (28% vs. 4%) anomalous Z-band configurations were found in the eccentrically trained subjects compared to control subjects. The structural changes were not typical of damage per se, but were interpreted as reorganization representing the regenerative activity of adapting fibres and acting as a defence mechanism against damaging tension during eccentric contractions. Researchers hypothesised that adaptations would provide better ability of the muscle fibres to stretch and recoil, reducing risk for mechanical damage and maintaining optimal overlap between actin and myosin filaments at rest (Friden *et al.*, 1983a;Friden, 1984).



Although distance runners do hill training incorporating both uphill and downhill, football players and some sprinters use downhill training alone as a form of assisted running to increase running speed. No scientific evidence has been published on the mechanisms of 'assisted' sprint training, but mechanisms may involve adaptations to the nervous system such as more synchronized motor unit activation, anticipatory firing and increased motor unit recruitment. It is also hypothesized that 'assisted' sprint training may improve joint stability and thereby allow greater transmission of force even when running on a horizontal track (Jakalski, 1998). 'Assisted' training may eventually lead to a faster stride frequency and an increase in stride length. Importantly, no reports exist of increased frequency of injuries in sprinters doing downhill training.

Numerous studies have tested chronic eccentric exercise and shown that muscle pain subsides after the first exercise bout and is not exacerbated by frequent training (Byrnes *et al.*, 1985;Friden *et al.*, 1983a;LaStayo *et al.*, 2000;Stupka *et al.*, 2001). Therefore muscle soreness should not been seen as a harmful aspect of eccentric exercise, but only as an acclimatization to a new training stimulus. The apparent risk of muscle injury due to recurrent downhill running raises concerns and care should be taken to ensure good running style. However, downhill training should not be totally disregarded due to unsubstantiated claims, and further research into downhill running is definitely warranted.

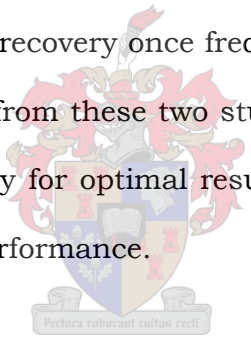
2.1.8 High intensity training

Endurance athletes often incorporate some form of high intensity training into their routine (Billat, 2001a;Billat, 2001b). This varies according to their specific needs, but may include hill training, fartlek sessions and track work. These sessions are designed to increase running power as well as enhance the aerobic energy system at high intensities, which is a result of the fairly prolonged duration of each interval.

Hill training is probably the most renowned form of high intensity training among athletes. Some world-class middle distance athletes credit their performances to rigorous hill training (Martin & Coe, 1997). Surprisingly little research has been done on hill training and the mechanisms behind this infamous training technique. Most current hill training techniques are based on coaches' knowledge and hearsay evidence. A few physiological studies have dealt with the metabolic costs and energy demands of an uphill run (Minetti *et al.*, 2002;Pringle *et al.*, 2002;Staab *et al.*, 1992), but none have looked at the muscular adaptations that occur with regular hill training.

2.1.9 Training Frequency

In the search to optimize training results, several studies have investigated the training frequency that would produce the greatest improvement. Most studies investigating strength training have found that a training frequency of 2-3 times per week is most beneficial (Carroll *et al.*, 1998;DeMichele *et al.*, 1997;Pollock *et al.*, 1993). To find the most efficient frequency of eccentric resistance training, Sorichter *et al.* (1997) compared three different training frequencies. They found that 2-3 training sessions per week produced greater increases in isometric MVC and less muscle damage after 7 weeks of training than less frequent training (Sorichter *et al.*, 1997). Lastayo *et al.* (2000) increased eccentric training frequency progressively during an 8-week study from 2 times per week to 5 times a week during the fifth week. During the sixth week of training subjects began to experience muscle fatigue (LaStayo *et al.*, 2000), possibly indicating accumulated fatigue or insufficient recovery once frequency increased to 5 times a week. Combining the information gained from these two studies, it seems eccentric exercise of at least 2 times a week is necessary for optimal results, but that more than 3 sessions per week could be detrimental to performance.



Confirmation for the above conclusion was also provided by Busso *et al.* (2002) who measured changes in maximal oxygen consumption and maximal power output in sedentary subjects who completed 3 high intensity interval cycling sessions per week for 8 weeks. After a 1 week rest period, subjects increased training frequency to 5 consecutive days each week. The higher training frequency resulted in an increase in magnitude and duration of the fatiguing effect from a single training session, as assessed by a 5 min performance test after every training session. Five sessions a week also decreased exercise-induced adaptation assessed by the maximal gain in performance as tested after the training interventions. Researchers concluded that muscles need 48 hours to recover after high intensity exercise, and more frequent high intensity training may be counterproductive (Busso *et al.*, 2002). However, the results should be treated

with caution since this study investigated sedentary subjects and the results can therefore not be directly applied to already well-trained athletes.

Billat *et al.* (1999) examined the addition of high intensity interval training to the normal training programme of well-trained runners. Endurance runners trained at the velocity of maximum oxygen consumption (vVO_2max) either once or three times per week for 4 weeks. One high intensity training session per week improved vVO_2max and running economy significantly, while 3 sessions per week had no positive effect (Billat *et al.*, 1999). To my knowledge this is the only study specifically investigating optimal training frequency in already well-trained endurance athletes, and it would seem that similar to untrained subjects, a too high frequency of training could have a disadvantageous effect. In recent literature investigating high intensity training techniques in well-trained endurance athletes, 2 high intensity training sessions per week seems to be the norm (Creer *et al.*, 2004;Laursen *et al.*, 2002;McMillan *et al.*, 2005;Roels *et al.*, 2005;Smith *et al.*, 1999).

In summary, 2 training sessions would probably constitute the best frequency of high intensity training. In contrast to the non-invasive assessments by the abovementioned studies, Carrol *et al.* (1998) compared the effects of different training frequencies by assessing changes in myosin heavy chain (MyHC) content of biopsies from the *vastus lateralis.* Although no significant inter-group differences were found after 6 weeks of resistance training (Carroll *et al.*, 1998), this may not be the best objective method to compare different training protocols, due to the relatively slow turnover of sarcomeric MyHC. Nonetheless, other parameters of muscle responses to training may more clearly indicate differences in response to different training protocols. In the following 2 sections I will discuss fibre type and its ability to transform and then satellite cells and their ability to respond to signals.

2.2 SKELETAL MUSCLE FIBRE TYPE

2.2.1 Characteristics

Human skeletal muscle consists of muscle fibres that differ in metabolic and contractile properties. Some fibres are equipped for oxidative metabolism while others are more efficient at glycolytic energy production. The difference in intrinsic capacity of myosin adenosine triphosphatase (mATPase) can be used to type muscle fibres histochemically through enzyme mATPase staining intensity (Meijer & Elias, 1976). Fibres can also be characterized by the isoforms of specific contractile and regulatory proteins they express (Schiaffino & Reggiani, 1996), most commonly through myosin heavy chain isoforms (Billeter *et al.*, 1980). On these grounds fibres are classified as oxidative type I, oxidative type IIa and glycolytic type IIx fibres. In this thesis I will follow the following convention: when fibre type was assessed histochemically or immunohistochemically isoforms will be named Type I, IIA, IIB; when expressed through gel electrophoresis to separate MyHC isoforms, the isoforms will be named type I, IIa, IIx. Type I fibres, also termed slow fibres, use mainly oxidative metabolism and contain large amounts of capillaries and mitochondria for efficient oxygen transport and phosphorylation respectively (Ingjer, 1979a;Wroblewski & Jansson, 1975). Type IIA fibres can produce ATP easily through either oxidative or glycolytic means or both, with the ratio between these pathways depending on exercise intensity. Fast type IIB-fibres function predominantly anaerobically to create immediate energy and maximal acto-myosin interaction for powerful contractions (Pette & Spamer, 1986). A greater proportion of slow type I fibres are found in endurance athletes compared to sedentary controls who have a greater percentage of type IIB fibres (Jansson & Kaijser, 1977).

2.2.2 Fibre type transformations

Initially, fibre type was believed to be almost exclusively determined by genetic makeup of the individual (Jansson & Kaijser, 1977;Komi *et al.*, 1977). In the last few decades, studies have proven that fibre type is able to transform to some extent in response to

various stimuli. The conversion from type IIB to IIA fibres has been observed repeatedly in response to different modes of exercise (Campos *et al.*, 2002;Hather *et al.*, 1991;Jurimae *et al.*, 1996;Kofotolis *et al.*, 2005) including eccentric exercise (Hather *et al.*, 1991). Type IIA fibres can also be converted "back" to type IIB fibres during detraining or denervation (Burnham *et al.*, 1997;Staron *et al.*, 1991).

After decades of research it still remains controversial whether type II fibres can be converted to type I fibres (Baumann *et al.*, 1987;Ingjer, 1979b;Jansson & Kaijser, 1977;Nuhr *et al.*, 2003;Short *et al.*, 2005;Thayer *et al.*, 2000). The inconsistency in results may be due to inherent methodological variability. Fibre type proportion has been shown to vary between biopsy sites and within the same muscle sample for distinct sections (Elder *et al.*, 1982;Lexell *et al.*, 1985). The different methods of determining fibre type proportions may also influence results. With histochemistry, fibres are counted individually to determine proportions, while with electrophoresis biochemistry, the relative amounts of different isoforms are measured, which can be influenced by fibre size. Therefore the second method will inadvertently produce somewhat different results in comparison with the first if fibre hypertrophy occurs only in a particular fibre type. Despite these differences, MyHC proportions have been proven to accurately reflect the contractile and metabolic properties of skeletal muscle (Pette *et al.*, 1999;Sant'ana Pereira *et al.*, 1995;Smerdu & Erzen, 2001).

Conversion from type I fibres to type IIx fibres has been observed after denervation but may take up to 20 months (Burnham *et al.*, 1997). This may imply that type I to type II transformations do occur, but take much more time and more extreme interventions than transformations between fast twitch fibre isoforms.

However, some of the strongest evidence for the conversion between fibre types in response to exercise is the presence of hybrid fibres (Putman *et al.*, 2004;Schantz, 1986;Schantz & Dhoot, 1987;Smerdu & Erzen, 2001). These fibres contain more than one MyHC isoform in each fibre as shown through single fibre analysis of MyHC content (Sant'ana Pereira *et al.*, 1995;Smerdu & Erzen, 2001). Studies of hybrid fibres indicate that there may in fact be a continuum between fibre types as follows: MyHC I – IIc – IIa – IIab – IIb (Sant'ana Pereira *et al.*, 1995;Schantz & Dhoot, 1987). Hybrid fibres probably reflect fibres that are in transition between expressing only the one or the other MyHC isoform (Putman *et al.*, 2004;Smerdu & Erzen, 2001). However, some authors argue that the occurrence of hybrid fibres is an adaptation to provide a more finely tuned variety of functional properties (Stephenson, 2001;Wu *et al.*, 2000). Fig. 2.2.2 illustrates the transition of fibre types along the continuum.

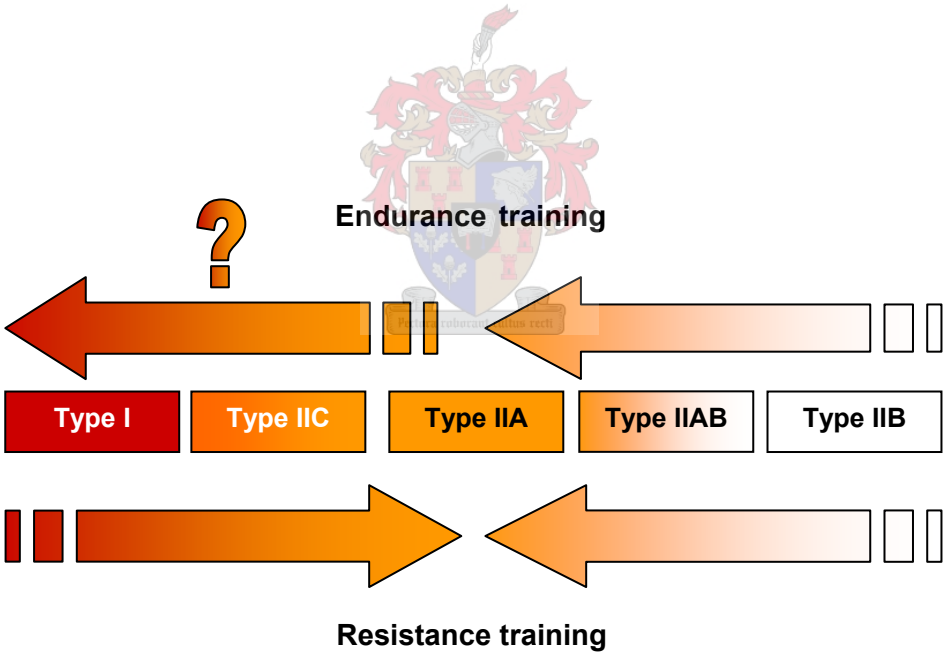


Fig. 2.2.2 ***Skeletal muscle fibre type transitions in response to exercise training***

2.2.3 Fibre type changes with eccentric training

Very little research has explored the changes in fibre type proportions that occur as a result of eccentric exercise training. There are six relevant studies, of which 5 investigated fibre type transitions after eccentric weight training (see in Table 2.2). Paddon-Jones *et al.* (2001) observed that maximum intensity eccentric weight training decreased the proportion of histochemically-typed slow twitch fibres and increased the proportion of fast twitch Type IIB fibres. This research is in contrast to the other studies that found eccentric resistance training increased Type IIA fibre proportions similarly to concentric resistance training (Friedmann *et al.*, 2004;Hortobagyi *et al.*, 2000). Paddon-Jones e*t al* (2001) specifically used fast eccentric contractions, which may explain the conversion to a faster muscle phenotype. It raises the question of how fibre type proportion may react to other forms of eccentric training such as eccentric ergometry.

Table 2.2 ***Changes in muscle fibre type proportions in response to eccentric training***

Study	Subject details	Biopsy site	Training type, Intensity, frequency	Muscle fibres type change
Hortobagyi *et al,* 2000	6M + 6F	*Vastus lateralis*	Eccentric weight training Maximal eccentric effort 3 x week, 12 weeks	+7% type IIA -11% type IIX
Hortobagyi *et al*, 1996a	7M	*Vastus lateralis*	Eccentric weight training Maximal eccentric effort 3 x week, 12 weeks	+12% type IIA - 7% type IIB
Friedmann *et al*, 2004	7M	*Vastus lateralis*	Eccentric overload weight training 30% eccentric 1RM 3x week, 4 weeks	+8% type IIA
Paddon-Jones *et al,* 2001	7M	Biceps Brachii	24 **fast** maximal eccentric reps 3 x week, 10 weeks	- 15% Type I + 7% Type IIX
	6M	Biceps Brachii	24 **slow** maximal eccentric reps 3 x week, 10 weeks	No difference
Raue *et al*, 2005	6M	*Vastus lateralis*	Eccentric weight training 80% concentric 1RM 3 x week, 4 weeks	+ 11% more hybrids MHC-I/IIa + MHC-IIa/IIx
Friden *et al,* 1983	15M	*Vastus lateralis*	Eccentric cycle ergometry Until severe fatigue 3 x week, 8 weeks	+8% Type IIC

Friden *et al.* (1983a) observed an increase in the proportion of type IIC fibres after 8 weeks of eccentric cycle ergometry. This result is similar to an increase in MyHC Ic and IIax hybrids observed recently by Raue *et al.* (2005) after 4 weeks of eccentric weight training. As the proportions of other fibre types did not decrease significantly, it is unknown in which direction these fibres were changing. Nonetheless, it is the first study to investigate the appearance of hybrid fibres in response to eccentric exercise and thus it is a valuable addition to the literature. At present the study by Friden *at* al (Friden *et al.*, 1983a) seems to be the only study that has investigated fibre type transition in response to eccentric ergometry. This study provides inconclusive, yet promising results of fibre type transition, and thus justifies further research in models other than weight training.

Fibre type switching is only one of many possible responses to eccentric training. In the next sections I will discuss myogenic responses to exercise. First a basic outline of our current understanding of satellite cells will be provided.

2.3 SATELLITE CELLS

During development of skeletal muscle, muscle precursor cells (mesodermal somatic cells) proliferate and differentiate into myoblasts (mononucleated muscle cells expressing myogenic markers). Several myoblasts fuse together to form multinucleated myotubes. Myotubes differentiate further and develop contractile properties to become post-mitotic multinucleated muscle fibres. Some of the muscle precursor cells do not differentiate to form myotubes, but remain mononucleated mitotic cells. They lie dormant (quiescent), scattered between the mature muscle fibres. This population of undifferentiated muscle precursor cells is known as satellite cells. They are positioned under the basal lamina of

the muscle fibre, but separated from the muscle fibre by the sarcolemma (Hawke & Garry, 2001).

Satellite cells can be activated to re-enter the cell cycle. Satellite cells proliferate and daughter cells may form new satellite cells that return to the dormant condition or fuse with mature muscle fibres. Despite this mechanism for replacement of the dormant satellite cell population by their daughter cells, the number of satellite cells, which is highest in post-natal muscle, diminishes with age (Hill *et al.*, 2003;Morgan & Partridge, 2003).

Progressive overload of skeletal muscle, such as resistance training or chronic stretching, results in muscle growth (Goldspink, 1999). These interventions may cause muscle damage, ranging from tears in the sarcolemma, basal lamina and connective tissue, to damage within the contractile and cytoskeletal proteins of the muscle fibre (Armstrong *et al.*, 1991). This myotrauma stimulates release of growth factors and cytokines that initiate a cascade of regeneration events, including the activation of satellite cells and other processes, eventually leading to muscle repair, regeneration and hypertrophy. In the following sections I will discuss some of the different stimuli which activate satellite cells and whether they are sufficient to prompt satellite cells incorporation into adult skeletal muscle tissue, as well as the role of satellite cells in different models of exercise.

2.3.1 Satellite cells and hypertrophy

Muscle hypertrophy is the increase in muscle size attributed to an increase in the amount of contractile proteins and thus the number of myofibrils within each fibre. With overloading of muscle, as occurs with resistance exercise, muscle fibres increase in size to enable overall superior force production whilst reducing the strain on individual myofibrils and the cytoskeletal structures attached to them.

Muscle hypertrophy is brought about by enhanced protein synthesis, leading to the increase in contractile proteins. Increases in transcriptional activity, leading to enhanced protein synthesis can be controlled by myonuclei. However, protein synthesis is mediated mainly through post-transcriptional regulation before, during and after translation (Booth *et al.*, 1998).

Each myonucleus in a multi-nucleated muscle fibre can only control a limited region of cytoplasm within the muscle fibre, called a myonuclear domain (Cheek, 1985;McCall *et al.*, 1998). With resistance training, individual myonuclei increase their transcriptional activity, and consequently their myonuclear domain will enlarge. As hypertrophy continues, myonuclear domain size will increase gradually (Kadi *et al.*, 2004a) and eventually reach a maximum volume. Current evidence suggests that at this stage, individual myonuclei within the muscle fibre have reached their maximum level of transcriptional capacity (Allen *et al.*, 1999). The control of the myonuclear domain size is not well undertsood.

For hypertrophy to persist beyond this restriction, an increase in the number of myonuclei per muscle fibre is required (Edgerton & Roy, 1991;Kadi & Thornell, 2000). Additional nuclei can be supplied by activation, proliferation and subsequent migration of satellite cells (Kadi *et al.*, 2004b). When satellite cells are sufficiently activated, they divide mitotically into daughter cells. While some of these daughter cells remain outside the sarcolemma as the next generation satellite cells, others migrate and fuse with enlarging muscle fibres (Allen *et al.*, 1999). Evidence of the mechanisms for these processes and the factors influencing these processes are accumulating rapidly, especially due to an increasing number of cell culture experiments. This will be reviewed in more detail in sections 2.4 and 2.5, whilst the focus of the next sub-section will be on evidence gained from studies in human subjects, which are far fewer.

Increases in myonuclear number in human studies

Several studies have demonstrated that the number of myonuclei per muscle fibre is greater in highly trained power-lifters than in untrained subjects (Eriksson *et al.*, 2005;Kadi *et al.*, 1999a;Kadi *et al.*, 1999b). Although these studies provided evidence for the addition of myonuclei, they were cross-sectional in design and reflected long-term adaptations over several years, with no investigator control over the various factors that might have influenced the finding. The direct evidence for satellite cell incorporation and myonuclear addition in response to exercise-induced hypertrophy is limited in human studies. Several studies have demonstrated a failure to increase myonuclear number significantly in hypertrophy (Hikida *et al.*, 2000;Kadi *et al.*, 2004b). The extent of hypertrophy may determine whether an increase in myonuclear number occurs or not. When Kadi & Thornell (2002) induced a 36% increase in mean cross-sectional fibre area through resistance training, large increments in both satellite cell and myonuclear numbers were observed (Kadi & Thornell, 2000). Similarly, when testosterone supplementation invoked extensive hypertrophy, an increase in satellite cell number and a proportionate increase in myonuclear number was observed (Sinha-Hikim *et al.*, 2003). These are the only two human studies to provide evidence of satellite cell incorporation into hypertrophied muscle, despite their different experimental design. Kadi & Thornell (Kadi & Thornell, 2000) studied changes in the *trapezius* muscle of women after 10 weeks of resistance training. This is in contrast to 20 weeks of testosterone supplementation in men without any training intervention in the other study mentioned above (Sinha-Hikim *et al.*, 2003). These differences imply that the extent of hypertrophy may be the most important determinant of increases in satellite and myonuclear numbers, rather than mechanical events. Nonetheless, there is evidence that satellite cells are affected by exercise. In a recent study, Kadi *et al.* (2004b) showed proliferation of satellite cells after 30 and 90 days of resistance training. However, there were no changes in the myonuclear number during the training period, implying that activated satellite cells were not incorporated into myofibres. Myonuclear domain increased

throughout the training period, but returned to baseline values after 90 days of detraining (Kadi *et al.*, 2004b). It is likely that although satellite cells were activated through exercise training, the extent of muscle hypertrophy was not enough to warrant their fusion with adult muscle fibres. The activation and subsequent deactivation of satellite cells implies that a threshold in myonuclear domain size was not reached. Alternatively, factors promoting migration were absent.

Table 2.3 ***Human studies measuring myonuclear number after hypertrophy***

Study	subjects	intervention	muscle	Satellite cell number	Myonuclei number	Myonuclear domain	Fibre cross-sectional area
Hikida *et al, 2000*	9 elderly men	16 weeks resistance training	*Quadriceps*		NS	NS	↑ 30%
Kadi *et al,* 2004b	15 young men	90 days resistance training	*Quadriceps*	↑ 31%	NS	↑ ~15%	↑ 17%
Kadi & Thornell, 2000	9 women	10 weeks resistance training	*Trapezius*	↑ 46%	↑ 70%		↑ 36%
Sinha-Hikim *et al*, 2003	12 men	20 weeks testosterone supplementation	*Quadriceps*	↑ 100%	↑ 44%		↑ 32%

NS, not significant

2.3.2 Satellite cells and Hyperplasia

A separate and controversial mechanism of muscle growth is hyperplasia. Hyperplasia is defined as the increase of muscle fibre number. Animal studies have shown the occurrence of hyperplasia. Longitudinal fibre splitting was observed in avian muscle in response to 28 days of chronic stretch overload (Antonio & Gonyea, 1994;Gonyea *et al.*, 1977). In cats subjected to weight training for 19-46 weeks, a 19% increase in muscle fibre number was observed, presumably through fibre splitting (Gonyea *et al.*, 1977). These increases in fibre number have, to date, not been confirmed in human studies comparing trained and untrained men (MacDougall *et al.*, 1984;McCall *et al.*, 1996) or

after a strength training intervention (McCall *et al.*, 1996). Currently, the only evidence for muscle hyperplasia in humans is the absence of differences in fibre area in highly trained and control subjects despite differences in arm girth. This indirectly suggest that there are more fibres present in the trained subjects compared to the controls (MacDougall *et al.*, 1982).

Recently, Kadi & Thornell (1999) suggested the occurrence of hyperplasia after resistance or endurance training in women. Subjects performed 3 training sessions a week for 10 weeks, whereafter a muscle biopsy was taken. The proportion of muscle fibres expressing neonatal or embryonic MyHC isoforms changed from none at baseline to above 3% after training for both groups. The authors observed the appearance of myotubes and small muscle fibres expressing embryonic and neonatal myosin heavy chain isoforms. It was concluded that some proliferated satellite cells fused together to form new muscle fibres. Fig. 2.1, reprinted from Kadi & Thornell (1999), illustrate their findings.

Embryonic and neonatal MyHC isoforms are expressed in muscle during development and regeneration. These isoforms are established markers for the initial stages of muscle fibre development (Butler-Browne & Whalen, 1984;Whalen *et al.*, 1990). Some authors hypothesize that the expression of these isoforms may also be considered evidence for new muscle fibre formation (Antonio & Gonyea, 1993;Kadi & Thornell, 1999;Kennedy *et al.*, 1988). In agreement with this theory, small sized muscle fibres have been observed in muscle samples from trained athletes and after training interventions (Alway *et al.*, 1989;Appell *et al.*, 1988;Friden *et al.*, 1983a;Kadi *et al.*, 1999a;MacDougall *et al.*, 1982). These data support the occurrence of hyperplasia, most likely through the fusion of satellite cells to form myotubes. However, in the absence of additional evidence such as the expression of developmental MyHC isoforms, there could be other explanations for small fibres.

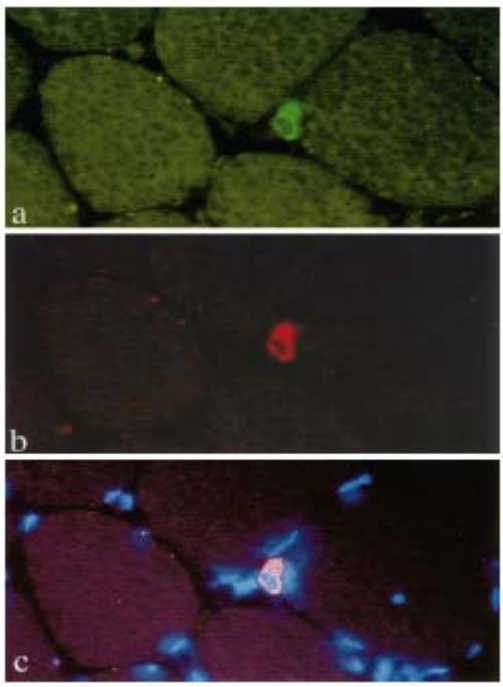


Fig. 2.1 ***Identification of myotubes in skeletal muscle:*** The presence of myotubes in female trapezius muscle after strength training as shown by Kadi & Thornell (1999). Co-staining with monoclonal antibodies against embryonic and neonatal MyHC isoforms demonstrate myotubes stained positively for neonatal MyHC (a), embryonic MyHC (b) and both mentioned isoforms (c)

2.3.3 Satellite cells after one exercise bout

To gain a better understanding of the mechanisms of muscle adaptation to exercise, it is necessary to examine the immediate changes after a single exercise bout. Although numerous animal studies have investigated satellite cell activation after a bout of exercise, only a handful of studies have focused on responses in human subjects.

Crameri *et al.* (2004) studied satellite cells' activation after a single session of eccentric resistance training with biopsies taken immediately, 2, 4 and 8 days post exercise. After the high intensity eccentric exercise bout, satellite cells were activated and beginning to differentiate, as assessed by positive staining for myogenin or developmental MyHCs, but did not show evidence of terminal differentiation and were not incorporated into existing myofibres. When exercise-induced damage has been severe enough to inflict necrosis of some myofibres, small regenerating fibres were present at 120 h, indicating satellite cell

differentiating and fusion despite the fact that only one bout of exercise was performed (Darr & Schultz, 1987). However, after this bout of eccentric running in rats, the authors suggested that the number of satellite cells activated is far greater than that required to replace the small number of necrotic fibres (Darr & Schultz, 1987). In another rat study, the number of activated satellite cells increased within the first few days after mechanical injury had induced necrosis, reached a plateau at 4 days post injury, and subsequently declined towards baseline values by day 7 (Hurme & Kalimo, 1992). I hypothesize that, in the absence of severe damage, repeated bouts of exercise are necessary to sustain activation and induce fusion of satellite cells into myofibres, unless severe damage occurs.

2.3.4 Satellite cells in eccentric and concentric exercise

The extent of muscle damage is particularly severe following eccentrically biased exercise. Studies that have compared damage induced by eccentric or concentric resistance training using qualitative electron microscopy, indicated that muscle fibres exhibit more severe disruption after eccentric exercise (Gibala *et al.*, 1995;Gibala *et al.*, 2000;Newham *et al.*, 1983). This is in agreement with findings that eccentric exercise causes greater delayed onset muscular pain than concentric exercise (Fitzgerald *et al.*, 1991). The severe pain and myofibre disruption after eccentric exercise is associated with the appearance of inflammatory mediators that are activated after eccentric but not concentric exercise. Increased cytokines such as LIF and IL-6 in circulation and muscle as well as increased transcription of pro-inflammatory genes in muscle tissue was seen (Chen *et al.*, 2003;Pedersen *et al.*, 1998). The inflammatory response to exercise is discussed in more detail in section 2.4.1.

It is plausible that the greater damage inflicted during an eccentric exercise bout may evoke a more aggressive myogenic response than seen after concentric exercise. Alternatively, the mechanisms of muscle repair and growth may be quite different

between these two modes of exercise, with concentric exercise using means other than damage to activate the process, i.e. without early activation of satellite cells. Although a few exercise studies have investigated satellite cell activation in response to eccentric resistance training (Crameri *et al.*, 2004) and predominantly concentric resistance training (Kadi *et al.*, 2004b;Kadi & Thornell, 2000), no one has investigated the differences between these two modes of exercise. This is surprising, as numerous studies have investigated the difference between the effects of eccentric and concentric exercise on hypertrophy and muscle strength (Friedmann *et al.*, 2004;Hortobagyi *et al.*, 1996b;Hortobagyi *et al.*, 1996a;Komi & Buskirk, 1972;Smith & Rutherford, 1995). Therefore a void exists in the literature to explore in more detail the differences between muscle adaptation following concentric and eccentric exercise.

2.3.5 Satellite cell activation during running

Very few studies have determined the capacity of either uphill or downhill running to induce myogenesis. Malm *et al.* (2004) investigated inflammatory markers after a bout of uphill or downhill running. Surprisingly markers for granulocytes, T cells and leukaemia inhibiting factor were not significantly different between uphill, downhill and control groups. The authors concluded that inflammation is not compulsory for muscle adaptation (Malm *et al.*, 2004). I find their interpretation to be controversial as it has not been substantiated by other research groups and has been contradicted by several. However I will not discuss this further in my thesis as it is a complex topic, requiring a thorough review of immunology, which does not fall within the scope of this study. In the rest of this thesis, in an attempt to simplify peripheral issues, I will assume that severe exercise inflicts an inflammatory response.

Muscle damage and the subsequent inflammatory cell infiltration in response to a single session of downhill running (Eston *et al.*, 1995;Feasson *et al.*, 2002;Smith *et al.*, 1998) have both been shown to resemble those of eccentric resistance exercise (Chen *et al.*,

2003;Gibala *et al.*, 1995;Stupka *et al.*, 2000). The quoted studies showed evidence of myofibrillar disruption and increased levels of plasma creatine kinase, as well as muscle inflammatory markers on protein and mRNA levels. These responses may be forerunners of muscular adaptation after dynamic and resistance types of eccentric exercise (1 or 2 sessions) and possibly training (more sessions). To my knowledge, nobody has investigated the effect of prolonged downhill running on signs of myogenesis in human subjects. Animal studies have shown significant satellite cell activation after multiple sessions of downhill running (Darr & Schultz, 1987;Smith *et al.*, 2001).

Friden e*t al* (1983a) observed hypertrophy after chronic eccentric cycling in humans over a 10-week period. Although the muscle sample analysis did not focus on quantification of satellite cell responses, light microscopy showed an increasing number of fibres of different sizes. The authors suggested that this finding was indirect evidence of new fibre formation, further suggesting that this was brought about by satellite cell incorporation and longitudinal addition of sarcomeres, both as possible mechanisms of muscle hypertrophy (Friden *et al.*, 1983a). This is the only longitudinal study exploring muscular adaptation after 8 weeks of repetitive eccentric ergometry in human subjects. More research is required to gain a better understanding of the adaptations involved.

2.3.6 Satellite cell activation during endurance running

Kadi *et al.* (2004a) found no indication of satellite cell activation after a single bout of endurance cycling using immunohistochemical markers to identify satellite cell activation. In contrast, a training study measured increased satellite cell numbers after 14 weeks of endurance running in aged subjects (Charifi *et al.*, 2003). Since these are the only two studies looking at satellite cell activation after endurance training, more research is necessary to gain a better understanding of the myogenic responses initiated by satellite cell activation in response to various modes of endurance exercise.

2.4 THE INFLUENCE OF GROWTH FACTORS AND CYTOKINES ON SATELLITE CELLS

The following sections of the literature review will outline and discuss shortly some of the factors that influence satellite cells. In some instances they have been elucidated in models including exercise, whereas other evidence has been gained from cellular studies.

2.4.1 Selected immune system components implicated in the response to muscle damage

Exercise-induced myotrauma initiates an immune response which results in several different immune-muscle interactive processes. An early response is the influx of neutrophils into the damaged area. The neutrophils' influx could possibly be induced by increases in free radicals, chemokines and growth factors (Tidball, 2005). Neutrophils' primary function is to promote inflammation through the release of cytokines that attract further inflammatory cells (Tidball, 1995).

Damaged fibres secrete various agents such as HGF that attract macrophages towards the injury site (Kimura *et al.*, 1996). Macrophages secrete cytokines that can play a role in regulating the satellite cell pool (Cantini *et al.*, 1994;Cantini *et al.*, 1995;Cantini & Carraro, 1995). One study has highlighted the importance of macrophages in the early events of muscle regeneration. When transplanted satellite cells were treated with macrophage inflammatory proteins, satellite cell proliferation and differentiation was accelerated and enhanced. Alternatively, in the absence of macrophages, muscle regeneration in transplants was disabled completely (Lescaudron *et al.*, 1999). Although neutrophils and macrophages secrete numerous cytokines, in the next few sections I will focus only on LIF and IL-6 since cytokine responses are not a key focus of this thesis.

Leukaemia inhibiting factor

Leukaemia inhibiting factor (LIF) is a cytokine produced by both macrophages (Robertson *et al.*, 1993) and myoblasts (Sakuma *et al.*, 2000). LIF binds directly to myoblasts *in vitro* and stimulates proliferation without affecting myoblast differentiation or fusion (Austin *et al.*, 1992;Vakakis *et al.*, 1995). It is probably through this mechanism that LIF promotes muscle regeneration in murine models, as observed by increases in muscle fibre size and number after damage and LIF administration (Barnard *et al.*, 1994;Kurek *et al.*, 1996).

Interleukin-6

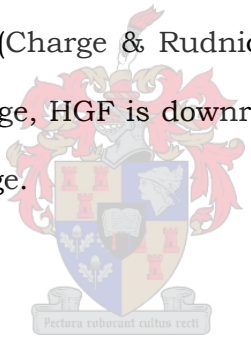
The cytokine interleukin-6 (IL-6) can be released by muscle fibres after exercise induced muscle damage (Tomiya *et al.*, 2004). Increased plasma IL-6 is, in part, also originally from the immune cells' inflammatory response to muscle damage resulting from exercise, rather than from circulating immune cells (Peake *et al.*, 2005). This cytokine may be involved in both satellite cell proliferation and fusion, as well as playing a role in inducing apoptosis of neutrophils and macrophages (Cantini *et al.*, 1995). According to Cantini *et al.* (1995) this is crucial for resolving inflammation during muscle repair. Whether or not the source of IL-6 in damaged muscle is from myonuclei or immune cells or both, IL-6 secretion would appear to be a self-limiting process.

2.4.2 Selected growth factors implicated in muscle growth and repair

Various growth factors also regulate satellite cell involvement in muscle repair and hypertrophy. Myofibres, satellite cells and inflammatory cells can produce some growth factors locally (autocrine and paracrine agents) while others are brought to the damaged area through the circulation (humoral agents).

Hepatocyte growth factor

Hepatocyte growth factor (HGF) is one of the most important growth factors involved in organ regeneration through its mitogenic and chemotactic properties (Zarnegar & Michalopoulos, 1995). HGF-receptor, *c-met*, is expressed by quiescent and proliferating satellite cells (Tatsumi *et al.*, 1998). Thus HGF could have a direct effect on the proliferation, and also indirectly the differentiation, of satellite cells (Bischoff, 1997). HGF stimulates satellite cells to enter the cell cycle and inhibits differentiation to maintain proliferation (Tatsumi *et al.*, 1998). HGF is released in quantities corresponding to the severity of muscle damage (Kimura *et al.*, 1996;Tatsumi *et al.*, 1998), and consequently it is hypothesized to function as a potent chemo-attractant for satellite cells (Bischoff, 1997). The main goals of HGF may be to increase the size of the satellite cell population through proliferation and to move them closer to the damaged area during early myogenesis by affecting migration (Charge & Rudnicki, 2004). Once enough myoblasts have been recruited to repair damage, HGF is downregulated, but this feedback has not yet been elucidated, to my knowledge.



Fibroblast growth factor

Similar to HGF, fibroblast growth factors (FGFs) are known to activate satellite cells' proliferation and to inhibit differentiation resulting in expansion of the local satellite cell population (Allen *et al.*, 1984;Yamada *et al.*, 1989). There are 20 FGF homologues that have been identified which exhibit different and redundant functions. FGFs bind directly to satellite cells through FGF-receptors (isoforms 1-4) during early stages of their activation (Sheehan & Allen, 1999). Animal studies suggest that FGF can induce proliferation and migration concurrently (Allen *et al.*, 1984;Bischoff, 1997;Boilly *et al.*, 2000).

Testosterone

Testosterone has been implicated as important for satellite cell activation and proliferation during hypertrophy. The administration of supra-physiological doses of testosterone promotes muscle hypertrophy and increases in satellite cell number along with a proportional increase in myonuclear number (Sinha-Hikim *et al.*, 2003). The mechanism by which testosterone stimulates myogenesis, though unclear, may involve direct communication with satellite cells, as suggested by androgen receptors present on satellite cells (Joubert & Tobin, 1995).

Transforming growth factor β

The transforming growth factor-β (TGF-β) family is a family of important cytokines regulating cell growth. In tissue culture experiments, it has been shown that TGF-βs are modulators of myoblast activity, inhibiting both proliferation and differentiation (Allen & Boxhorn, 1989). Immunohistochemistry of muscle samples has shown TGF-β accumulation immediately prior to inflammation in the junctions between the viable and necrotic portions of fibres (McLennan & Koishi, 1997). The authors hypothesized that this finding may be indicating a function in regulating immune responses. Fusing satellite cells and newly formed myotubes contained strong TGF-β immunoreactivity, providing evidence for their role in myoblast fusion.

A member of the TGF-β family, myostatin, has recently been identified as an inhibitor of muscle growth (McPherron *et al.*, 1997). Plasma myostatin levels are decreased during resistance training, indicating opposite modulatory effects of training on myostatin and muscle hypertrophy (Walker *et al.*, 2004). As recently reviewed by Charge & Rudnicki (2004), research suggests that myostatin regulates skeletal muscle mass, at least in part by inhibiting satellite cells' proliferation. However, whether or not myostatin affects satellite cells directly, is at present undetermined.

Insulin-like growth factor-1

Insulin-like growth factor-1 (IGF-1) has various physiological functions, including growth and development. Initially IGF-1 was thought to be an exclusively liver-secreted growth factor in response to growth hormone (GH) secretion. Eventually it became clear that extra-hepatic tissues such as skeletal muscle could release IGF-1 locally to pursue functions that are independent of GH (Jennische & Hansson, 1987). The actions of IGF-1 on muscle are many-fold. It enhances protein synthesis through upregulation of both transcription and translation and through improved amino acid uptake by myofibres (Fryburg *et al.*, 1995). Secondly, IGF-1 is involved in the proliferation and differentiation of satellite cells and their subsequent fusion either to form myotubes or to be incorporated into growing myofibres (Adams, 2002). Recently different isoforms of IGF-1 have been identified, of which two are expressed in skeletal muscle after exercise (Fig. 2.2). A third isoform, IGF-1Eb, is not expressed by skeletal muscle and appears to be active as an endocrine growth factor only (Bloor *et al.*, 2001).

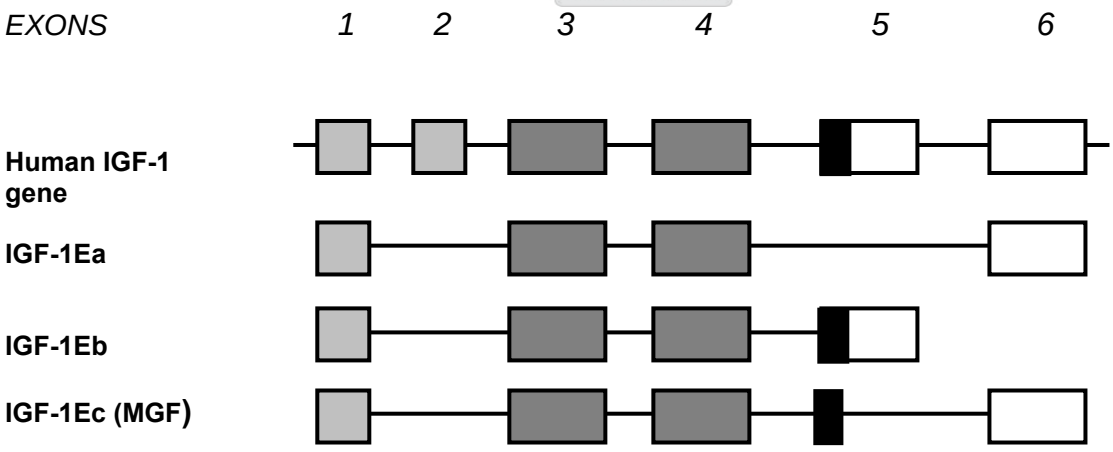
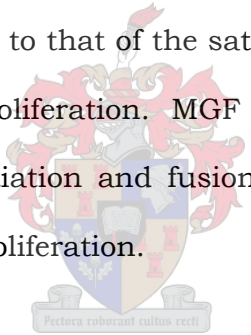


Fig. 2.2 ***IGF splice-variants:*** A schematic representation of the IGF-1 gene and its mRNA splice variants expressed in muscle. The black boxes denote the insert in exon 5 (49bp), which gives rise to the alternatively spliced MGF isoform (Hameed *et al.*, 2003).

i) Mechano growth factor

Mechano growth factor (MGF), also termed IGF-1Ec, carries an extra 49-basepair insertion (Fig. 2.4.2 presents a schematic illustration of the different IGF-1 splice variants). MGF is a smaller molecule than IGF-1 and has a shorter half-life, making it an effective paracrine/autocrine agent (Goldspink, 2003). MGF is observed in skeletal muscle after chronic stretch or resistance training (McKoy *et al.*, 1999). MGF is an extremely responsive and effective isoform, showing a 1000% upregulation in mRNA after 5 days of mechanical overload in rats (Owino *et al.*, 2001) and inducing a 25% increase in fibre cross-sectional area after two weeks of daily injection in animal models (Goldspink, 2002). Expression of MGF precedes expression of MyoD, a marker for satellite cell activation (Hill & Goldspink, 2003), evidence that may link MGF to the activation of satellite cells after a stimulus. In addition, after stimulation MGF upregulation follows a similar trend to that of the satellite cell marker M-cad, illustrating its association with enhanced proliferation. MGF has also been implicated in the prevention of satellite cell differentiation and fusion (Yang & Goldspink, 2002), which may explain the enhancement of proliferation.



ii) IGF-1Ea

IGF-1Ea is an autocrine and paracrine isoform that is similar to the main liver endocrine isoform of IGF-1. Over-expression of this isoform in mice with a tissue specific-transgene, has been shown to induce persistent hypertrophy without pathological changes in the muscle architecture (Musaro *et al.*, 2001). However, IGF-1Ea mRNA has been shown to be absent 2.5 hours after resistance exercise in humans despite upregulation of the MGF isoform (Hameed *et al.*, 2003). In a rat model subjected to mechanical damage, IGF-1Ea was slowly upregulated (from day 5 post-damage onwards) as the initial rapid expression of MGF mRNA subsided. This increase in IGF-1Ea corresponded to the drop in expression of markers for satellite cells' activation as indicated by m-cadherin immunostaining and MyoD mRNA (Hill & Goldspink, 2003). Hill and Goldspink

furthermore showed that MGF was upregulated only in damaged muscle, while IGF-1Ea mRNA was upregulated in stimulated and contra-lateral muscle, implying local and systemic control of IGF-1Ea expression. The delayed expression of IGF-1Ea may implicate involvement in myoblast differentiation and fusion (Hameed *et al.*, 2003).

iii) Human studies

Several studies have measured drastic increases in skeletal muscle IGF-1 mRNA expression after an acute bout (Bamman *et al.*, 2001) or several weeks of resistance training (Singh *et al.*, 1999), but did not differentiate between splice-variants. Hameed *et al.* (2003) have shown specifically the upregulation of MGF mRNA 2.5 hours after a single resistance exercise bout. No increase in IGF-1Ea or MyoD expression was observed at that time. This is feasible since 2.5 hours post-exercise is too early for the activation of IGF-1Ea, which is associated with satellite cell differentiation and fusion (Hameed *et al.*, 2003). In a subsequent study, 5 weeks of resistance training in elderly men increased mRNA of both MGF (163%) and IGF-1Ea (68%). Growth hormone administration, seemed to intensify both MGF and IGF-1Ea expression in muscle tissue but had no effect on MGF when administered unaccompanied by exercise (Hameed *et al.*, 2004). In contrast to Hameed *et al.* (2003) who found no change in IGF-1Ea 2.5 hours after resistance exercise, Psilander *et al* (2003) found a decrease in IGF-1Ea one and six hours after a resistance exercise bout. This might substantiate *in vitro* experiments that indicated IGF-1Ea involvement in myoblast fusion (Yang & Goldspink, 2002) which is a later event, although the time point is still early after the stimulus. In summary, during early stages of satellite cell activation MGF may be necessary for proliferation, while IGF-1Ea is downregulated to delay fusion and prolong the potential for satellite cell involvement in myogenesis. Psilander *et al.* (2003) found several inconclusive results (Non-significant tendencies) regarding responses of different IGF-1 isoforms, and attributed this to large inter-subject variation. Their subjects were young men classified as being habitually active without taking part in competitive sports. This stresses the importance of a

homogenous subject group regarding current fitness levels. The types of training in which subjects previously participated, and to which they are currently adapted, may also influence results.

Since it is still early in the history of research focussing in IGF-1 or MGF experiments in human subjects' muscle, it is not surprising that few studies have compared different modes of exercise. One study found IGF-1 increased significantly 48 hours after eccentric but not after concentric resistance exercise (Bamman *et al.*, 2001). This may be indicative of a greater myogenic response following more severe damage inflicted by eccentric exercise. Alternatively, mechanical forces may activate gene transcription without damage (Nader *et al.*, 2002). More research is necessary to gain a comprehensive understanding of the role of MGF after different types of exercise. Currently it seems that the only way approach this is by PCR analysis of MGF mRNA, since antibodies to the protein (human) is not commercially available.

2.5 SATELLITE CELL IDENTIFICATION

Satellite cells can be identified by their physical location at the periphery of mature muscle fibres outside the sarcolemma, but underneath the continuous basal lamina that surrounds the satellite cell and the associated muscle fibre. Definitive morphological identification can be done using an electron microscope. Satellite cells can also be identified and quantified using immunohistochemistry, where specific primary antibodies bind to secondary or tertiary fluorescent markers and are viewed under a fluorescence microscope. Hence, satellite cells can be recognized by the proteins they express. Markers that are not expressed by myonuclei are most useful in correct identification of satellite cells. Satellite cells can express different markers at different stages of their cell cycle, assisting in the quantification of total, activated or proliferating satellite cells. Several different surface proteins and transcription factors are used for satellite cell

identification. The quality of such a marker depends on its specificity of binding to satellite cells as opposed to other muscle or non-muscle structures. I will briefly discuss the markers that were chosen for use in my own study, while a more comprehensive list of other markers often used in other studies are shown in table 2.4.

Table 2.4 ***Expressed markers for satellite cell identification***

Expressed marker	Stage of expression	Example references
	Cell surface proteins	
M-cadherin	Quiescent, activated, proliferating, differentiating	(Irintchev *et al.*, 1994)
c-met	Quiescent, activated, proliferating, differentiating	(Cornelison & Wold, 1997)
N-CAM (CD56)	Quiescent, activated, proliferating	(Malm *et al.*, 2000)
Syndecan-3&4	Quiescent, activated	(Cornelison *et al.*, 2001)
CD34	Quiescent	(Beauchamp *et al.*, 2000)
	Transcription factors	
MyoD	Activated, proliferating	(Koishi *et al.*, 1995)
Myf5	Activated, proliferating	(Cooper *et al.*, 1999)
Myogenin	differentiation, fusion	(Andres & Walsh, 1996)
MRF4	differentiation, fusion	(Zhou & Bornemann, 2001)
Pax7	Quiescent, activated, proliferating	(Reimann *et al.*, 2004)
MNF	Quiescent, activated, proliferating	(Garry *et al.*, 1997)

N-CAM, nuclear cell adhesion molecule; MRF4, myogenic regulatory factor 4; MNF, myocyte nuclear factor.

2.5.1 CD56 / Leu19

The CD56 antibody recognizes the neural cell adhesion molecule (N-CAM), a cell-cell recognition glycoprotein expressed during early myogenesis and in adult muscle satellite

cells (Illa *et al.*, 1992;Schubert *et al.*, 1989). N-CAM is one of the most useful markers for satellite cells in biopsy samples of human muscle as it binds to quiescent as well as active satellite cells without binding to myonuclei (Malm *et al.*, 2000).

CD56 has been used successfully in several studies to quantify satellite cells in human subjects' muscle samples (Borg *et al.*, 1993;Higuchi *et al.*, 1999;Kadi *et al.*, 1999b;Malm *et al.*, 2000). Unfortunately this marker does not allow the distinction between quiescent and proliferating satellite cells.

2.5.2 M-cadherin

M-cadherin is a calcium-dependent cell adhesion molecule that is also expressed prominently during developmental skeletal myogenesis (Rose *et al.*, 1994). It plays a possible role during the alignment and fusion of myoblasts to enlarge myotubes (Cifuentes-Diaz *et al.*, 1995). M-cadherin is expressed in quiescent and activated satellite cells in animal models (Cornelison & Wold, 1997;Irintchev *et al.*, 1994). M-cadherin is strongly expressed by myoblasts during early stages of regeneration and downregulated after fusion of myoblast to myotubes (Irintchev *et al.*, 1994). In human muscle biopsy samples baseline M-cadherin levels have been used to identify the quiescent satellite cell population (Reimann *et al.*, 2004;Sajko *et al.*, 2004) as well as to measure satellite cell activation by its upregulation (Hill *et al.*, 2003). Similarly this marker cannot be used to accurately predict activation of satellite cells because it may stain both quiescent and proliferating satellite cells. Therefore changes in M-cad positively stained cells only provide evidence of increased proliferation relative to baseline numbers.

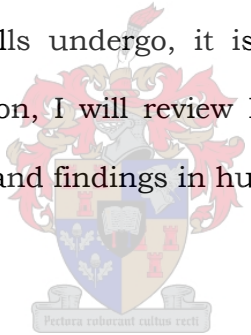
2.5.3 MyoD and Myogenin

MyoD is a member of the helix-loop-helix family of myogenic regulatory factors (see next section for details). In short, MyoD is expressed upon activation of satellite cells and

continues to be expressed during proliferation. MyoD immunoactivity is lost once satellite cell nuclei fuse to form myotubes (Koishi *et al.*, 1995).

Myogenin, also a muscle specific transcription factor, is implicated in cell cycle arrest and regulates phenotypic differentiation and subsequent cell fusion (Andres & Walsh, 1996). Myogenin is essential for myoblast differentiation and is therefore an good marker of satellite cell differentiation (Brunetti & Goldfine, 1990).

In summary, it is possible to identify satellite cells specifically as opposed to other nuclei, although some markers are not definitive. MRFs play an important role in satellite cell experiments as they can be used to determine the stage of development and as well as assisting numerical quantification of the changes in satellite cell population. To properly understand the stages satellite cells undergo, it is necessary to further discuss the MRFs. Therefore, in the next section, I will review MRFs with special reference to the scope of their expression over time and findings in human studies.



2.6 MYOGENIC REGULATORY FACTORS

Myogenic regulatory factors (MRFs) are basic helix-loop-helix transcription factors that play a large role, and in some cases are essential for the development of skeletal muscle. However they are important not only for embryonic and foetal development, but also postnatal growth and adult muscle repair. MRFs function as transcription activators due to their inherent DNA-binding properties, initiating transcription and regulating gene expression (Funk *et al.*, 1991).

Much knowledge of MRFs has been gained from experiments in transgenic mice where individual MRFs have been knocked out through embryonic stem cell technology. Homologous mice null for either MyoD (-/-) or Myf5 (-/-) develop normally (Braun *et al.*,

1992;Rudnicki *et al.*, 1992), but when both these factors are absent, no muscle formation occurs and muscle forming regions of the foetus are deficient in myogenic stem cells (Rudnicki *et al.*, 1993). Transgenic mice without myogenin (-/-) display normal myoblast populations, but lack functional muscle due to an absence of terminal differentiation of myoblasts (Hasty *et al.*, 1993). MRF4 null (-/-) mice develop normal muscle tissue but exhibit extensive upregulation of myogenin (Zhang *et al.*, 1995) indicating either that MRF4 function can be compensated for completely by myogenin, or that the actual role of MRF4 is to downregulate myogenin. In another study, constitutively expressed MRF4 can lead to normal myofibre formation in the absence of myogenin (Sumariwalla & Klein, 2001). It seems that the MRFs functions are to some extent overlapping and can thus compensate for shortcomings in one or another MRF.

Analysing upregulation of MRFs provides an effective method to study the time course of myogenesis. Most data about MRF expression has been gathered from cell culture and animal studies. Myf5 is present in quiescent satellite cells and may be responsible for maintaining the commitment of these cells to a myogenic lineage (Beauchamp *et al.*, 2000). Myf5 and MyoD may be necessary for the recruitment of quiescent satellite cells into the cell cycle to start proliferating (Cooper *et al.*, 1999). Accordingly, these two MRFs are the first to be expressed after exercise induced damage in rats (Armand *et al.*, 2003;Smith *et al.*, 1994). In addition, MyoD seems to be important for early differentiation and the transition to myogenin positive cells (Yablonka-Reuveni *et al.*, 1999). Myogenin expression coincides with cell cycle withdrawal and phenotypic differentiation of myoblasts in cell culture (Andres & Walsh, 1996). Studies in animals indicate that myogenin is essential for myoblast commitment to differentiation (Hirayama *et al.*, 1997). MRF4 expression does not occur in proliferating satellite cells but is restricted to the time around and after fusion into myotubes (Zhou & Bornemann, 2001).

Many discrepancies exist in the literature on the time course of the myogenic response, which makes it difficult to compare studies and draw conclusions. The activation and transition between stages of myogenic response differs between development, muscle regeneration and muscle growth (Creuzet *et al.*, 1998). In addition, responses vary between cell culture, animal and human models. For the purpose of this study, I will focus mainly on the myogenic time course in response to exercise in humans.

2.6.1 Time course of the myogenic response

Only very recently have studies appeared in the literature on MRF expression in adult humans. In general, studies have shown varying degrees of MRF upregulation after exercise or no increases at all. Therefore it is necessary to examine the study results, but also the protocols in detail.

Six studies used a single bout of resistance exercise and MRF mRNA was shown to peak 6 – 24 hours after loading (Bamman *et al.*, 2004;Bickel *et al.*, 2005;Hameed *et al.*, 2003;Psilander *et al.*, 2003;Willoughby & Rosene, 2003;Yang *et al.*, 2005). One study found large increases in mRNA for myogenin (440% 6h post exercise), with somewhat less elevation in MyoD (110% immediately post exercise), and MRF4 (120% 2h post exercise) after a resistance exercise bout. The training protocol stimulated the *vastus lateralis* by using 4 sets of 6-14 repetitions of leg press and knee extensor exercises to exhaustion (Psilander *et al.*, 2003). Bickel *et al.* (2005) analysed only MyoD and myogenin and at somewhat later time points, but mRNA increases for MyoD (83%) were still evident 12h post-exercise and myogenin mRNA (3-fold) peaked 24h after a resistance training bout (Bickel *et al.*, 2005).

Willoughby and Nelson (2002) examined both mRNA and protein levels of myogenin and MyoD after a resistance exercise bout which trained the *quadriceps* using 3 sets of 8-10 repetitions of leg press, squat and knee extension exercises at 75-80% 1RM. Protein and

mRNA levels increased immediately post exercise and were even higher at 6 hours post exercise. At this time mRNA levels were elevated 46% and 47% while protein expression increased 318% and 254% for MyoD and myogenin respectively (Willoughby & Nelson, 2002). The lower mRNA levels at comparable time points to the two aforementioned studies may be due to the previous resistance training experience of the subjects, although they did not engage in a consistent heavy resistance training programme. In contrast to these studies, Bamman *et al* (2004) observed no changes in MRF protein levels 24 hours after resistance loading, despite a similar protocol to Willoughby & Nelson's and a greater number of untrained subjects. A possible explanation may be that these authors observed a blunted myogenic response due to several, progressively more difficult familiarization sessions intended to prepare subjects for the full loading bout.

In the most thorough investigation into the time course of myogenic responses to exercise until now, acute bouts of exercise were followed by multiple biopsy sampling during the first 24 hours post-exercise. A resistance exercise session increased mRNA levels of MRF4 (4.5 fold 4 hours post), MyoD (5.8 fold 8h post) and myogenin (3.5 fold 12 h post) (Yang *et al.*, 2005). This is in agreement with previous studies showing evidence of myogenic gene transcription 2 - 6 hours post-exercise, with evidence of elevations remaining until 24 hours post-exercise (Hameed *et al.*, 2003;Psilander *et al.*, 2003;Willoughby & Nelson, 2002).

Responses to habitual resistance training may vary from those seen for acute exercise in unaccustomed subjects. After 10 weeks of resistance training to rehabilitate from short-term immobilization, hypertrophy was accompanied by an increase in myogenin protein expression while the other three MRFs did not show protein level differences from pre-immobilization (Hespel *et al.*, 2001). In the same study, a second group responded to resistance training plus creatine supplementation with an increase in MRF4, which correlated with a change in mean fibre diameter. In a similar study, myogenin, MyoD and

MRF4 protein and mRNA increased after 12 weeks of resistance training while no changes in Myf5 occurred (Willoughby & Rosene, 2003). Changes at protein and mRNA levels followed a similar trend. It is difficult to compare the results of these studies as neither specified the precise time point after the last training session when muscle biopsies were taken. Both studies used a progressive resistance training approach and baseline subject characteristics did not differ notably. The subjects in the second study trained at a greater intensity (9 sets x 6-8 repetitions at 85-90% 1RM including leg press, knee extension and knee curl) (Willoughby & Rosene, 2003) compared to the subjects in the study by Hespel *et al.* (4 sets x 12 repetitions knee extensions at 60% isometric 1RM)(2001). The greater training intensity may explain the greater myogenin response and continued upregulation of the other MRFs observed by Willoughby & Rosene (2003).

Bickel *et al.* (2005) investigated MRF responses after two successive resistance bouts separated by 48 hours. They found sustained and elevated levels of myogenin concentrations after the second bout, while MyoD increased after the first bout and subsequently declined (Bickel *et al.*, 2005). More research should clarify the MRF responses after prolonged training and whether or not damage is required for MRF expression. It is possible that MyoD is expressed in response to injury, but myogenin to other hypertrophic signals.

Lowe & Alway (1999) have shown that satellite cell proliferation is not necessary for the upregulation of MRF mRNA. Quails were exposed to gamma radiation to eliminate proliferating cells before their muscles were subjected to stretch overload. Despite the absence of satellite cells, MRF mRNA were upregulated similarly to the stretch overload-only group (Eppley *et al.*, 1993;Lowe & Alway, 1999). This study indicates that MRFs may have other functions in skeletal muscle apart from differentiation and fusion of satellite cells. In the next section I discuss the potential role of MRFs in different fibre types.

2.6.2 MRF expression in different fibre types

Kadi *et al.* (2004a) recently showed that increased myogenin staining after an endurance exercise bout (one-legged cycle ergometry) is restricted to myonuclei rather than to satellite cells. MyoD expression was absent at baseline and did not change with endurance exercise irrespective of exercise intensity. Nuclei expressing myogenin varied considerably in the small subject pool (n=5), with no expression in two subjects at low exercise intensity (40% VO_2max) and in two subjects at higher exercise intensity (75% VO_2max). In a recent study, a 30 minute bout of running at 75% VO_2max increased only MyoD mRNA (5 to 8 fold) but not Myf5, myogenin or MRF4 in several biopsies taken 8 – 12 hours after exercise (Yang *et al.*, 2005). These studies cannot be compared directly, as the exercise mode (cycling vs. running) and muscle phenotype (*vastus lateralis* vs. *gastrocnemius*) was different. Despite these differences, the two groups reached the same conclusion, citing changes in MRF mRNA expression as evidence for their involvement in transcriptional regulation of fibre type specific oxidative metabolic properties (Kadi *et al.*, 2004a;Yang *et al.*, 2005).

The aforementioned conclusion is based mainly on results from prior animal studies. Myogenin and MyoD have been implicated indirectly in regulation of fibre type characteristics because of the observation that MyoD mRNA is present in fast twitch muscle fibres and myogenin mRNA in slow twitch muscles (Dupont-Versteegden *et al.*, 1998;Hughes *et al.*, 1993;Voytik *et al.*, 1993). In addition, alterations in fast/slow fibre type distribution correspond with parallel alterations in MyoD/myogenin expression patterns (Hughes *et al.*, 1993). Another study by Hughes *et al.* (1997) showed the mice in which the MyoD has been disrupted, show a shift towards a slower phenotype in fast muscles. In human *vastus lateralis* muscle, Willoughby and Nelson examined MRF responses to resistance training. Myogenin mRNA expression correlated with type I and IIa MyHC mRNA whereas MyoD mRNA with type IIx MyHC mRNA (Willoughby & Nelson, 2002).

In animals, pharmacologically induced adaptation (administration of clenbuterol or thyroid hormone) has been shown to increase the proportion of type IIx MyHC and concurrently increase MyoD and decrease myogenin mRNA expression (Mozdziak *et al.*, 1998). This shift to a faster phenotype and the concurrent increase in MyoD, strengthens the theory that MyoD and myogenin play roles in the regulation of fibre type specificity. The roles of MRFs in regulation of fibre metabolic properties are still unclear and need to be further investigated. More research on a protein level, instead of on gene transcript level, may also provide new insights into these processes.

2.7 SUMMARY

Eccentric exercise and especially chronic eccentric exercise remains a research field with many unanswered questions. More research could reveal potential advantages of eccentric training for sport performance and rehabilitation from injury and disease. Similarly, research on myogenesis in human skeletal muscle is a developing field fuelled by an increasing amount of literature related to muscle repair and exercise induced adaptation. However, the behaviour of satellite cells in response to chronic exercise is a largely unexplored terrain. An increased knowledge of the satellite cell activation, proliferation and incorporation into adult myofibres and the various factors that influence these processes, could open new avenues for developing treatment strategies and training principals. In this study, I will combine into a training programme the demands of high intensity running and eccentric exercise for already-trained athletes. I will investigate possible performance changes as well as molecular changes brought about by the intervention. My aims are explained in the next section.

CHAPTER 3: AIMS OF THE STUDY

The intention of the present investigation was to study selected aspects of muscle adaptation following 4 weeks of either eccentrically of concentrically biased high intensity treadmill training in well-trained, but not elite, subjects.

Muscle adaptation was studied with the following aims:

a) To investigate whether or not uphill or downhill interval training influence race performance and maximum exercise test results similarly in well-trained middle distance runners,

b) To determine the extent of satellite cell proliferation in skeletal muscle in response to repeated sessions of eccentrically or concentrically biased high intensity training,

c) To measure the expression of the myogenic regulatory factor, myogenin, at protein level in skeletal muscle biopsies taken after two sessions and after 4 weeks (10 sessions) of high intensity training,

d) To determine the effect of repeated sessions of eccentrically or concentrically biased training on muscle fibre-type composition and muscle fibre cross-sectional area,

With reference to the parameters above

e) To determine differences between the early versus late phases of the skeletal muscle response to high intensity training, by comparing biopsies taken early (after two training sessions) and later (after 10 sessions, 4 weeks)

f) To determine whether the mechanisms of muscle adaptation differ between uphill and downhill running by comparing selected parameters of the myogenic responses, the fibre area and fibre type composition.

CHAPTER 4: METHODOLOGY

4.1 STUDY DESIGN

4.1.1 Subjects

13 moderately trained, but not elite male road runners were recruited from the Stellenbosch University student population. Strict inclusion and exclusion criteria were chosen: 1) Only subjects who trained between 35 and 70 km/week were included in the study, 2) all subjects were unaccustomed to eccentric exercise, 3) no subjects participated in intensive hiking or hill walking, 4) all subjects were free from cardiovascular or inflammatory conditions, 5) none had suffered musculoskeletal injuries in the 3 months prior to starting the study and, 6) subjects had to be between 18 and 25 years of age. The Ethics Committee of research administration Sub-committee C (for research including human subjects) of Stellenbosch University granted approval for the study. Written informed consent was obtained from subjects before their inclusion in the study. Subjects were assigned to either an uphill training group (UP, n=7) or to the downhill training group (DH, n=6) according to their current training volume in order to create two matched groups.

4.1.2 Study outline

The protocol included three phases. Phase I consisted of baseline performance testing and baseline muscle biopsy sampling. Phase II included 10 high intensity interval training (HIT) sessions completed over a 4 week period. This phase included two further muscle biopsies, one after 2 training sessions and one after the last training session. Phase III included post-training performance testing. The study design is illustrated in Fig. 4.1.

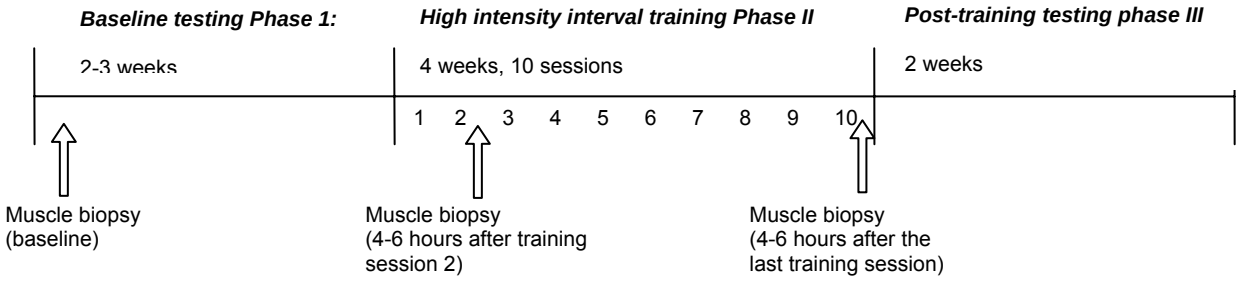


Fig. 4.1 ***Schematic presentation of the study design***

4.2 PERFORMANCE TESTING

Within three weeks preceding the commencement of the training intervention and within two weeks after the completion of training, subjects underwent a battery of performance tests. The testing protocol was kept exactly the same, except for the omission of the familiarization incremental exercise test after the training phase.

4.2.1 10 km time trial (TT)

Subjects completed a simulated 10 km road race before and after testing. An out-and-back route was mapped out (using a calibrated "meter-wheel") with a gradual incline during the first 3 km and consequently a gradual decline during the last 3 km. Total 10 km, times as well as the 5 km split times, were recorded. This was done to measure possible performance differences between the predominantly uphill 5 km out-run and the predominantly downhill 5 km back-run. To simulate a competitive race, subjects always completed their time trial together with other athletes (n > 10); route markers indicated each kilometre and water points were placed on the route. Fig. 4.2 displays the elevation changes of the 10 km route as measured with a heart rate monitor with altimeter (s710, Polar, Sweden). Unfortunately, outdoor 10 km TT performance is subject to the influence of uncontrollable factors such as ambient temperature, wind direction and wind strength, which may confound results and therefore 2 laboratory performance tests were also included.

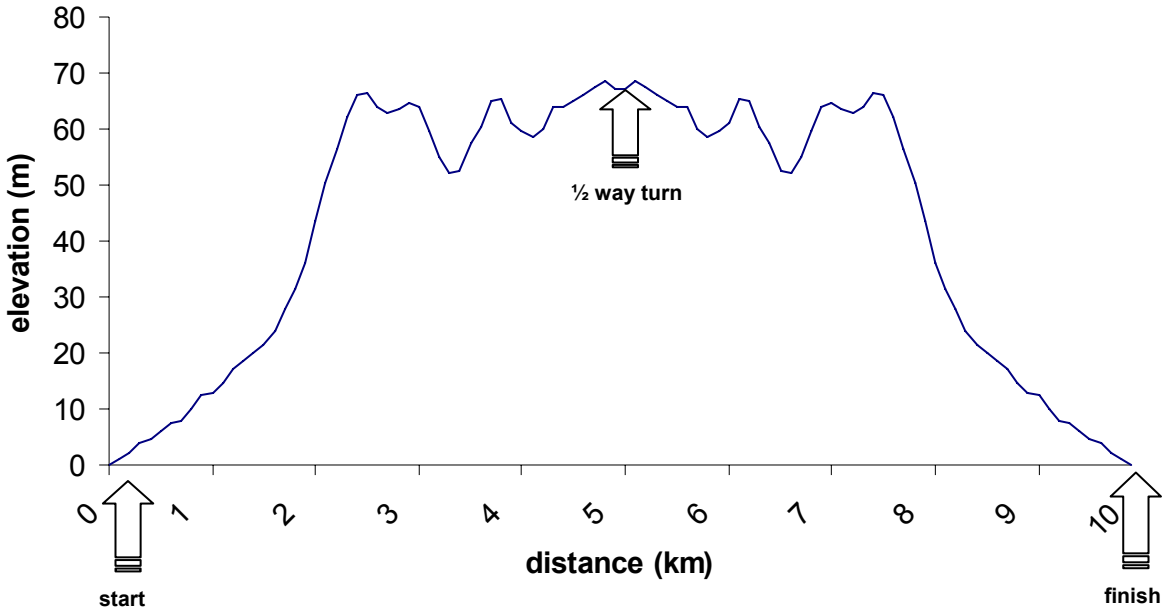


Fig. 4.2 ***Variation in elevation along the 10 km time trial route***

4.2.2 Incremental exercise test until exhaustion

Upon their first visit to the exercise laboratory, subjects were familiarized with the treadmill (Runrace, Technogym, Italy). An introductory horizontal incremental exercise test was completed to accustom subjects with the test protocol. During the next two to three weeks subjects completed another two incremental exercise tests.

Incremental exercise test protocol:

Subjects completed one incremental exercise test on a level treadmill and one on an inclined treadmill (+5% gradient). Tests were completed on days when subjects were in a relatively rested condition, i.e. they did not perform a hard training session on the previous day. A minimum of one day of recovery was given between exercise tests. Before the onset of the test, each subject warmed up at the subject's chosen pace for as long as needed, but for at least eight minutes. Subjects wore light running apparel and a heart rate monitor with chest band (s710, Polar, Sweden). The test started at a standard speed for all subjects and increased by 0.5 km/h every 30 seconds (Table 4.1).

Table 4.1 ***Test protocol for continuous incremental exercise tests to exhaustion***

workload	time	0° Gradient	5° Gradient
1st	30 sec at	12.0 km/h	10.0 km/h
2nd	30 sec at	12.5 km/h	10.5 km/h
3rd	30 sec at	13.0 km/h	11.0 km/h
4th	30 sec at	13.5 km/h	11.5 km/h
5th	30 sec at	14.0 km/h	12.0 km/h
nth	*x* sec at	Final km/h	Final km/h

The test continued until the subject was unable to complete the final speed. Subjects were verbally encouraged to continue as long as possible. The exact time when subjects discontinued running was noted. The test was seen as successful in eliciting a maximal exercise response when subjects reached two of three criteria: 1) a plateau in oxygen consumption (VO_2), 2) a maximal heart rate within 10 beats per minute of their age-predicted maximum, and 3) a respiratory exchange ratio greater than 1.10. A plateau in VO_2 was defined as no increase in VO_2 despite an increase in HR. Age-predicted HRmax was defined as the subject's age in years subtracted from 220. Peak treadmill speed (PTS), the maximum speed a subject reached on the test, was calculated by the following equation:

$$\text{PTS} = \text{Final completed speed} + 0.5 \text{ km/h} \times \frac{\text{seconds sustained on final speed}}{30\text{sec}}$$

Breath-by-breath gas analysis

Gas exchange data (oxygen uptake, VO_2; and carbon dioxide output, CO_2) were collected breath by breath with an automated gas analyser (OxyCon Pro, Jaeger, Germany). Before each exercise test, the pneumotachograph was internally calibrated with 2 L of air. The gas analyzers were calibrated with atmospheric O_2 (20.93%) and a known concentration of CO_2 (4.97%). VO_2max during each incremental exercise test was taken as the highest average VO_2 for any 30 second interval.

4.3 TRAINING INTERVENTION

4.3.1 Groups

Subjects were divided into either a downhill (6) or an uphill (7) training group. Uphill and downhill training groups were well matched for training distance, 10 km TT time, VO_2max and peak treadmill speed. During baseline the training volume was assessed by interview and questionnaire. Additionally, in a sub-group of 8 subjects, training volume was assessed by a 7-day training logbook. Training volume compared well between questionnaire and 7-day training log (see Appendix *i*). There were no significant difference between groups for age, body mass or height. All baseline data are presented in Table 4.2.

Table 4.2 ***Descriptive characteristics and baseline performance parameters***

	Uphill training group	**Downhill training group**	**All subjects**	**Range** (min –max)
n =	7	6	13	
Age (yr)	21.3 ± 1.5	19.5 ± 1.5	20.5 ± 1.7	18 - 23
Body mass (kg)	69.1 ± 5.3	70.7 ± 5.4	69.8 ± 5.4	63 - 78
Height (cm)	178.5 ± 5.6	178.3 ± 3.5	178.4 ± 4.4	169 - 184
Training distance (km/week)	49.3 ± 11.5	48.5 ± 9.8	49.9 ± 10.3	35 - 64
VO_2max $_{flat}$ (ml/kg/min)	62.1 ± 4.7	62.7 ± 4.0	62.4 ± 4.1	53.5 - 68.0
VO_2max $_{uphill}$ (ml/kg/min)	63.5 ± 5.9	63.5 ± 2.0	63.5 ± 4.5	53.8 - 71.5
PTS $_{flat}$ (km/h)	20.1 ± 1.1	20.0 ± 0.9	20.1 ± 1.0	18.6 - 21.5
PTS $_{uphill}$ (km/h)	16.7 ± 0.7	16.7 ± 1.0	16.7 ± 0.8	15.4 - 17.5
10 km time trial time (min)	38.77 ± 1.91	39.59 ± 1.91	39.15 ± 1.88	35.93 - 41.23

Values are means ± SD; *n,* no. of subjects; VO_2max, maximum oxygen consumption; PTS, peak treadmill speed; 10 km time: seconds converted to decimal

4.3.2 High intensity training protocol

Both groups completed 4 weeks of high intensity interval training (HIT) consisting of 10 high intensity interval training sessions. In 4 subjects (2 downhill, 2 uphill) more than 10 sessions were completed (11-12) to ensure correct timing of the post-training muscle biopsy, depending on the schedule of the doctor. HIT training was completed in the exercise laboratory on electronically braked treadmills (Runrace, Technogym, Italy; Trackmaster, JAS, USA). During the first and last weeks of training, 2 HIT sessions were completed, while during the 2nd and 3rd weeks of training, 3 HIT sessions were completed. HIT sessions were separated by a minimum of 48 hours to allow for recovery between sessions. Subjects were asked to maintain their usual training programmeme as far as possible during the 4 weeks and not to commence any new training routines. All training was monitored during the 4-week period by a daily training log. Heart rate was measured continuously during each HIT session. To compare heart rate (HR) responses during training between subjects, maximum HR during a training session was chosen as the HR reached during the 4th training interval. Similarly recovery heart rate was taken as the HR at the end of the rest interval following the 4th interval (shown in Fig. 4.3). Subjects warmed up thoroughly before each HIT session and took a cool-down run afterwards.

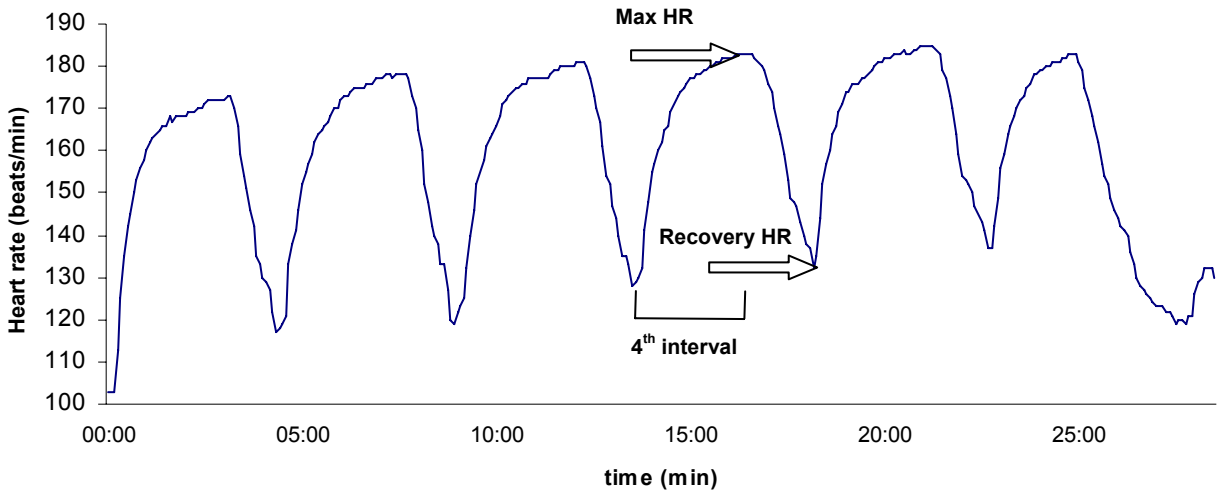


Fig. 4.3 ***Cardiovascular response to a typical high intensity training session***

Uphill training

Uphill training was performed at a 5% incline at 95% of the peak treadmill speed attained during the gradient incremental exercise test (PTS_{uphill}). Subjects trained in intervals lasting 2 min 30 s to 3 min each and rested for 2/3 of the interval time, depending on the individual subject's response to training. Subjects were encouraged to continue until exhaustion or until 6 intervals were completed. Once a subject could complete six intervals, each lasting 3 min, comfortably (only in 2 subjects), the interval length was increased to challenge the subject to keep progressing.

Downhill training

Downhill training was performed at a 10% decline at 95% of the peak treadmill speed attained during the horizontal incremental exercise test (PTS_{flat}). During each training session subjects completed up to 6 intervals lasting 3 minutes with 1 minute of rest between intervals. Once a subject appeared comfortable at a training speed and could complete 6 intervals, the training speed was increased for the next training session. This was done several times for each subject to increase the maximum heart rate response as much as possible and to provoke a progressive training stimulus.

4.3.3 Non-invasive measurements during training intervention

Perceived exertion

A subjective assessment of exertion for each training session was recorded immediately after the session using Borg's rating of perceived exertion (RPE) scale (Borg, 1970). Briefly, the scale consists of 15 values, starting at 6 and counting upwards to 20, with increasingly severe stages of fatigue ascribed to them. Subjects were asked to rate their level of exertion through expressing it as a number on the Borg scale. Fig. 4.4 shows the scale used.

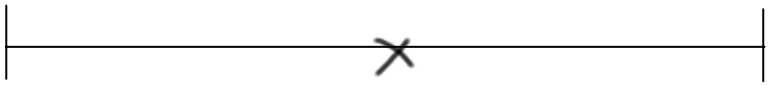
6	**No exertion at all**
7	**extremely light**
8	
9	**very light**
10	
11	**light**
12	
13	**somewhat hard**
14	
15	**hard**
16	
17	**very hard**
18	
19	**extremely hard**
20	**maximal exertion**

Fig. 4.4 ***Rating of perceived exertion (Borg, 1970)***

Perceived Pain

Muscle soreness was measured using the Visual Analogue Scale (VAS). Subjects rated their perceived muscle pain of the lower extremities, with emphasis on the *quadriceps* muscle group, before every training session. Instead of providing a numerical value to describe the severity of pain, subjects were instructed to mark a cross on a 100 mm continuous line as an indicator of leg pain. This is illustrated in Fig. 4.5.

Indicate the extent of pain experienced by the leg muscles, especially the *quadriceps* muscle group, through a mark on the line below:



no pain ***very severe pain***

Fig. 4.5 ***The Visual Analogue Scale***

The position of the mark on the line was measured and converted into a score for leg pain. The VAS has previously been used effectively to quantify pain inflicted by DOMS (Boyle *et al.*, 2004;Chen *et al.*, 2003;Cleary *et al.*, 2002;Slater *et al.*, 2005).

4.3.4. Biopsy sampling

Three muscle samples were removed from each subject during the study. The first was a baseline biopsy taken from the left leg when subjects were in a rested state (did not train immediately before the biopsy, and no hard training on the previous day). A second biopsy was taken approx 4-6 hours after the second training session from the right leg. A final biopsy was taken after the last training session from the left leg, approximately 1 cm proximal to the baseline biopsy incision site. Because of scheduling difficulties with numerous subjects training on the same day, the time of biopsies after exercise was not identical, with most biopsies performed within 4-6 hours after training (in two cases biopsies were taken < 2 hours after training). Subjects rested at least two days after each muscle biopsy (no training at all). Biopsies performed on the same leg were at least 6 weeks apart, ensuring that no carry-over effect of the previous biopsy would confound results. One subject did not consent to a second biopsy, but he again agreed to a third biopsy. Due to possible variation in fibre type distribution from superficial to deep and proximal to distal sites, the baseline and 4 weeks training biopsy were extracted from approximately the same location on the left leg, 1 cm from the pre-biopsy scar and at similar needle depth.

Biopsy procedure

A licensed medical practitioner experienced in the technique performed percutaneous needle biopsies. Biopsies were taken under local anaesthetic (2% lignocaine HCL, Xylotox, Adcock Ingram ltd, RSA) at constant depth from the mid-portion of the *vastus lateralis*, from the lateral side. A small incision was made through the skin, dermis and muscle fascia. A 5 mm stainless steel biopsy needle (Stille, Sweden) was inserted into

the muscle and a 100-150 µg muscle sample extracted using suction, as previously described (Evans *et al.*, 1982). A piece of muscle was selected for immunohistochemistry and was mounted on cork and surrounded in tissue freezing medium (Jung, Germany) and quickly frozen in liquid-nitrogen-cooled isopentane. The remaining muscle was snap frozen in liquid nitrogen for subsequent gel-electrophoresis and immunoblotting. All samples were stored at –80° C pending analysis.

4.4 LABORATORY ANALYSIS

4.4.1 Immunohistochemistry for identification of satellite cells (SC)

For immunohistochemistry, only subjects for whom suitable muscle sections could be sectioned for all three time points, were included in analysis. Transverse serial sections (8 µm thickness) of the mounted muscle biopsy samples were cut using a cryostat microtome (CM1100, Leica, Germany) at -20 to -23 ºC, mounted on poly-lysine coated slides and stored at -20º C. Within 2-3 months sections were co-stained for M-cadherin (quiescent and activated SC) and CD56 (quiescent and activated SC) as indicated below. Briefly, slides were fixed in acetone for 2 minutes and left to air dry. Sections were then blocked (to prevent non-specific binding) with 5% horse serum for 25 min at room temperature. Thereafter sections were incubated with CD56 mouse monoclonal antibody (1:100, in PBS, 170 mg/L, Dako Cytomation, M7074) for 4 hours at 4 °C. Afterwards slides were washed in phosphate buffered saline (PBS, pH 7.4, see Appendix) for 15 min and incubated in biotinylated secondary antibody (donkey anti mouse, 1:500, in PBS) at room temperature for 40 min. The slides were then washed in PBS for 20 min and subsequently incubated with Fluorescein Streptavidin, (1:500, Vector laboratories, USA) for 30 min at room temperature. Slides were then washed in PBS for 20 min and subsequently incubated with M-cadherin rabbit polyclonal antibody (1:50, in PBS, 200 µg/ml, Santa Cruz, SC-10734) overnight at 4 °C. After washing slides in PBS (15 min) they were incubated with biotinylated secondary antibody (donkey anti rabbit, 1:1200, in PBS) for 40 min at room temperature. The slides were washed again in PBS for 20 min. Thereafter sections were incubated with Texas Red Streptavidin (1:500, in PBS, Vector Laboratories, USA) for 30 min at room temperature. Then Hoescht was added to sections (50 µg/ml, 1:100) for a further 10 min. Sides were washed in PBS for 20 min prior to being mounted with cover slips using fluorescent mounting medium (Dako Cytomation, USA). Slides were stored at -20 °C until microscope analysis.

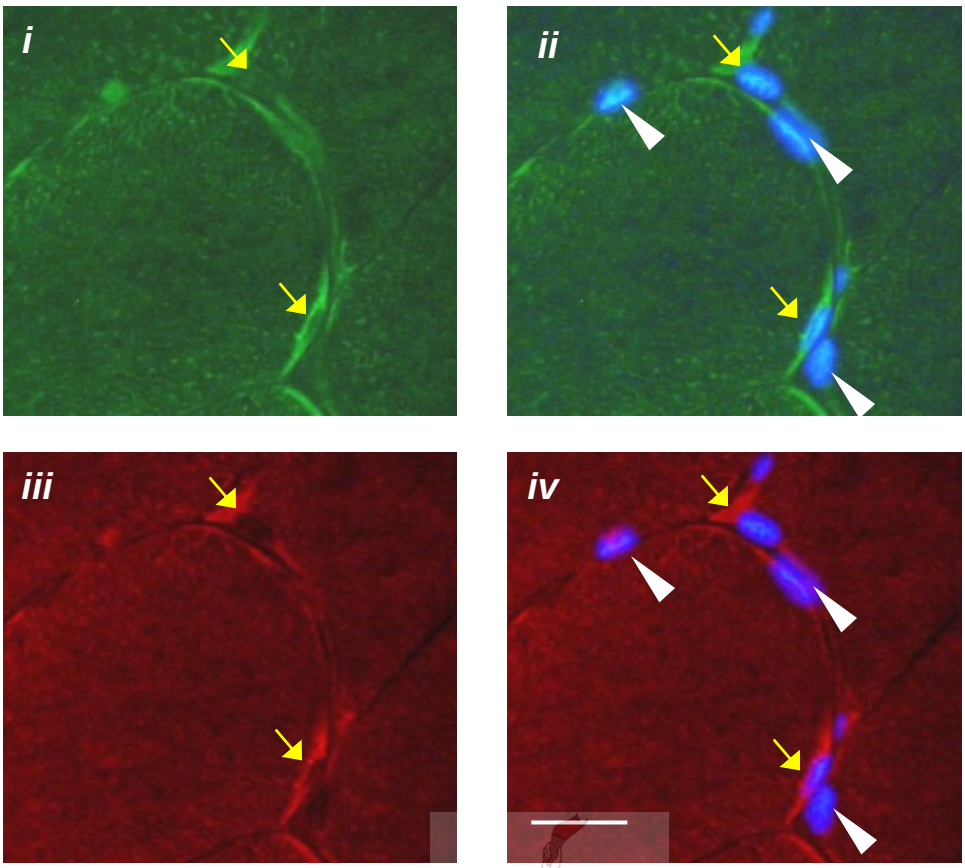


Fig. 4.6 ***Identification of satellite cells in immunohistochemically stained muscle sections.*** Sections *i – iv* show the same magnified portion of a muscle section stained for *i)* CD56, *ii)* CD56 & Hoescht (all nuclei), *iii)* M-cadherin and, *iv)* M-cadherin & Hoescht (40x). Yellow arrows identify satellite cells, which can be distinguished from myonuclei (within the sarcoplasm, white arrow heads) by the surrounding fluorescent staining and its position outside the sarcolemma. Bar = 20 μm

Fluorescent microscope analysis

Three sections per biopsy were stained, of which the two that stained best (least background staining) were analyzed. Slides were viewed through a fluorescence microscope (Eclipse E400, Nikon, Japan) using fluorescein (CD56 expression), Texas Red (M-Cad expression) and Hoechst (nuclei) filters. Photos were taken at 40X magnification with integrated digital camera (Nikon DXM1200, Japan) using Nikon ACT-1 software (version 2.12, Japan). For every muscle biopsy sample, 10-12 fields (5-6 per section) were photographed and subsequently examined using digital imaging software (Simple PCI, Compic Inc., USA).

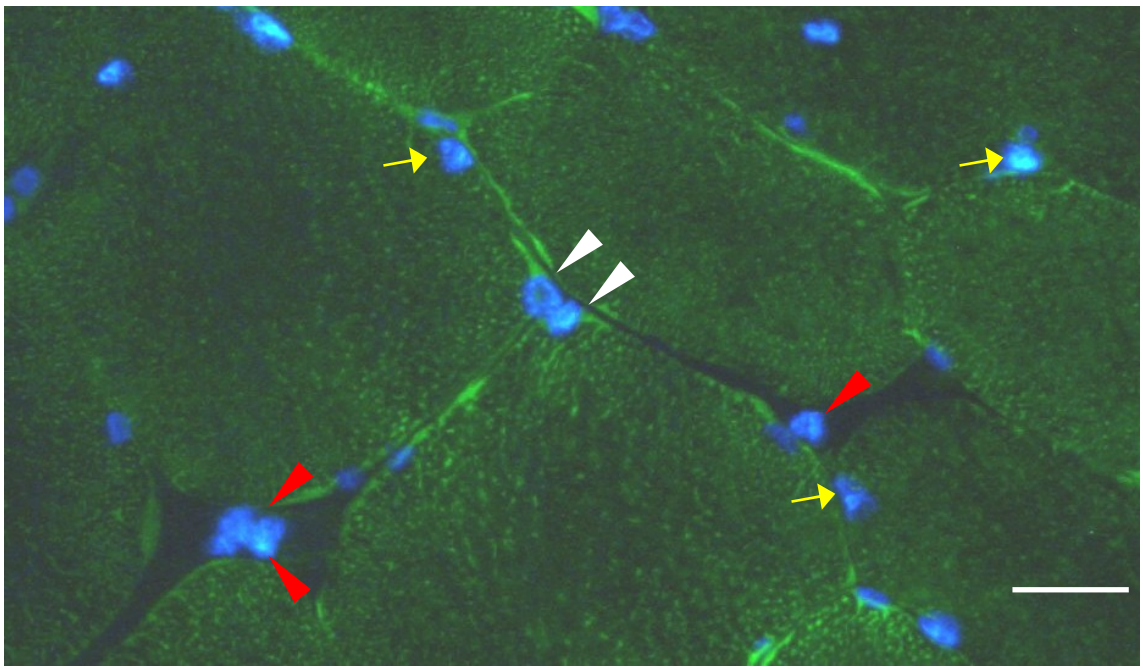


Fig. 4.7 ***Distinguishing between interstitial nuclei, satellite cell nuclei and myonuclei:*** The figure depicts a section stained with CD56 (green) and Hoechst (blue, all nuclei). Yellow arrows identify myonuclei, white arrows satellite cells, and red arrowheads interstitial nuclei. Bar = 20 µm

Satellite cells were identified as nuclei positioned between the sarcolemma and basal lamina that stained positive for CD56 or M-cadherin (Fig. 4.6). The numbers of satellite cells and total nuclei (satellite cells + myonuclei) as well as fibres were counted in each field of view. Only nuclei that could clearly be distinguished as lying in the interstitial space were excluded (example in Fig. 4.7). All the nuclei and fibres within the field of view were counted (including partial fibres on the edge of the field of view and their nuclei). There were typically between 15 and 20 fibres per view. Satellite cells were expressed relative to the *total* number of nuclei (satellite cells included) or relative to the number of fibres in the field:

Satellite cells / nucleus = Satellite cells / (Satellite cell nuclei + myonuclei)

Satellite cells / fibre = Satellite cells / fibre number per field

Fibre cross-sectional area was determined by tracing the periphery of the basement membrane of each fibre that appeared as a complete fibre in the field of view, and calculating the circumference with SIMPLE PCI Software (COMPIC Inc). Software was calibrated using a control image with known dimensions under the same magnification as immunohistochemistry photos (40x). For each biopsy the areas of ~70 fibres were measured (Fig. 4.8).

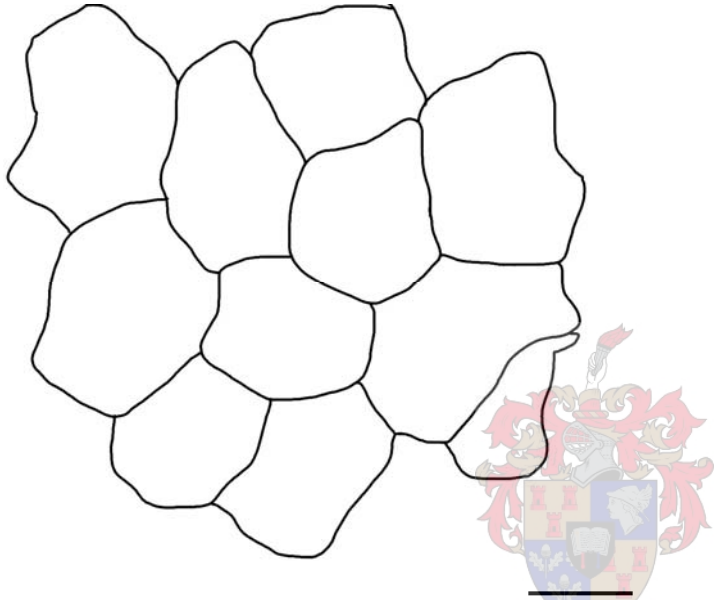


Fig. 4.8 ***Determination of fibre cross-sectional area***
originally 40x magnification; bar = 50 µm

4.4.2 Biochemistry

Muscle homogenate preparation for electrophoresis

Muscle samples were crushed in liquid nitrogen and weighed while still frozen. Muscle samples ranged from 20 – 60 µg. Thereafter muscle was homogenized in an eppendorf with 10 µl/g ice-cold lysis buffer (see Appendix) using a custom-built eppendorf homogenizer. Homogenates were left on ice for an hour hereafter they were centrifuged at 14000 rpm for 25 min at 4 C°. The supernatant was collected and stored in aliquots at -80 C°.

Muscle total protein concentration

Supernatant samples were assayed for total protein concentration by the Bradford method with bovine serum albumin (BSA) as standard (Bradford, 1976). Bradford reagent consisted of Coomassie Brilliant Blue G250 (0.2 g/l), 95% ethanol (5%), phosphoric acid (8.5% m/v) and dH_2O. Bradford reagent was filtered and stored at 4° C in a light impenetrable bottle. BSA standard (5 g/l) was made up in dH_2O and diluted into aliquots ranging from 0.05 to 3 g/l and stored at -80 C°. An ELISA plate reader (EL800, universal plate reader, BIO-TEK Instruments Inc, USA) was used for spectrophotometry. Muscle supernatant samples were diluted 1:10 with dH_2O prior to analysis. For analysis, sample: reagent ratio was 1:60. After incubation for 5 min at room temperature, absorption was read at 595 nm. All samples were analysed in duplicate and protein concentration was between 12 and 18 µg/µl for all muscle samples.

Electrophoresis for MyHC isoform proportions

Supernatants of muscle samples were diluted to 1 µg/µl in sample buffer (see Appendix) and separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The method was adapted from Andersen (Andersen *et al.*, 1994), Talmadge & Roy (Talmadge & Roy, 1993), and Blough *et al.* (Blough *et al.*, 1996). Details of the composition of the separating and stacking gels can be found in the Appendix. Upper running buffer had twice the concentration of the lower running buffer (Upper = 100 mM Tris, 150 mM Glycine, 0.1% SDS). 5 µl of each sample was loaded into wells of a minigel electrophoresis system (Bio-Rad MP3, Bio-Rad Laboratories, USA). Baseline and 4 -week samples for the same subject were loaded in adjacent lanes to standardize run conditions. After loading of all the samples, 200 µl ß-mercaptoethanol was added to the upper running buffer. Electrophoresis duration was 24 hours, voltage constant 70 V and temperature 4 C°. Gels were stained with Coomassie Brilliant blue R250 for at least 5 hours. Thereafter gels were placed in fixing solution (40% methanol, 10% acetic acid) for an hour and left in destaining solution (10% methanol, 7% acetic acid) for 24 hours.

Myosin heavy chain isoforms were identified from their apparent molecular masses, with migration rate: I > IIa > IIb (Fig. 4.9). The proportions of MyHC isoforms were analysed using a densitometry software package (Cream 1D, MEM-EN-TEC, Denmark). The relative intensity of the different bands is each expressed as a percentage of the total of the two or three bands.

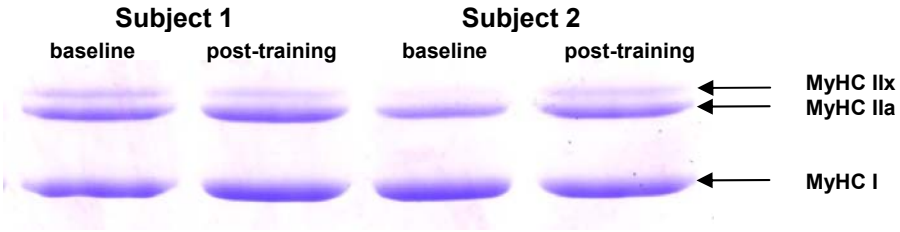


Fig. 4.9 ***Myosin Heavy chain isoform separation***

Western blotting for myogenin protein expression

Control experiments were run to determine an appropriate amount of protein to load and appropriate concentrations of antibodies that would produce the best results. 100 µg of total protein was diluted 1:2 in sample buffer (as before). Samples were separated by SDS-PAGE (constant 130 V, 80-90 min) in 10% poly-acrylamide minigels (Bio-Rad MP3, Bio-Rad Laboratories, USA) at room temperature. Proteins were transferred from gels at constant voltage (100 V, 60 min, 4° C) to polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories, USA) using a wet transfer system (Bio-Rad MP3, Bio-Rad Laboratories, USA). For each subject, all three consecutive biopsy samples were loaded in adjacent lanes. A C2C12 (mouse muscle myoblast cell line) sample known to be differentiated and expressing high levels of myogenin was run on every gel as control. Each gel contained samples from an uphill- and a downhill trained subject. A biotinylated protein ladder (Peqlab, Germany) was also run on every gel to ensure that the correct protein was blotted (myogenin = 36 kDA). Equal loading across lanes and equal transfer were

confirmed by staining all membranes with Ponceau S (0.1% w/v with 5% acetic acid (v/v)) after development.

Before immunoblotting, membranes were blocked against non-specific binding with 5% fat free milk in TBS-t (Tris-buffered saline: NaCl 0.88% w/v, KCl 0.02% w/v, Tris base 0.3% w/v, Tween-20 0.5%, all in dH_2O, pH 7.4) for 1 hour at room temperature. Membranes were incubated overnight in myogenin mouse monoclonal antibody (5% fat free milk, TBS-t, 1:100, 200 µg/ml, Santa Cruz, SC-12732) at 4 C°. After a TBS-t rinse protocol (2 x 7.5 min), membranes were incubated in secondary antibody (polyclonal rabbit anti mouse, 1:3000, 5% fat free milk in TBS-t) for 1 hour at room temperature. After rinsing (3 x TBS-t 5 min, 1x TBS 15 min) bands were visualized by chemiluminescence (Lumiglo reserve, KPL, USA) using a hypercassette (Amersham Biosciences, USA). Fig. 4.10 represents an example of a western blot membrane after development.

Band densitometry was performed using automated digitizing software (UN-SCAN-IT software, version 5.1, SILK SCIENTIFIC Corporation). The data for each subject are presented relative to the subject's own baseline sample, designated a value of 1. Baseline samples from each subject were also expressed relative to a specific subject who was designated a value of 100. This could be done by running the same samples on several gels and comparing their expression relative to each other afterwards.

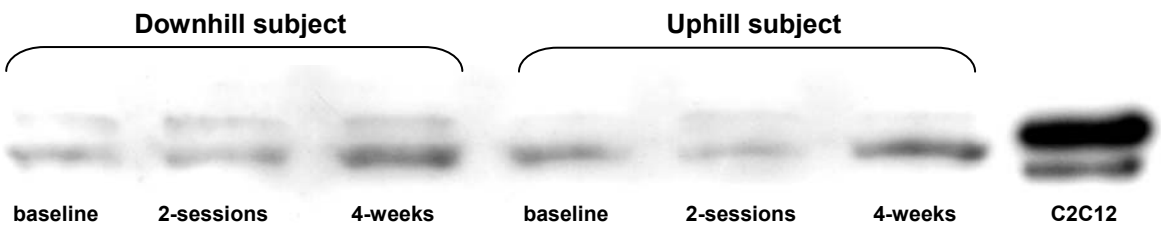


Fig. 4.10 ***Western Blot of muscle myogenin protein expression***

4.5. STATISTICAL ANALYSIS

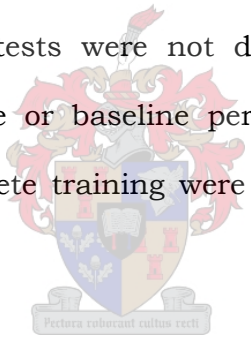
All statistical calculations were performed with a software package (Statistica, version 7.0, Statsoft Inc, USA). For all variables with two groups and with more than one time point, repeated measures ANOVAs were performed. Bonferroni tests were performed to determine post hoc significance. Residuals were plotted against normal probability to observe the distribution of data. If residuals were linearly distributed, tests were seen as valid, as was the case for all analyses. To compare two groups of independent data, Mann-Whitney U tests or Student's unpaired t-tests were used, depending on the distribution of the data. For some data (such as fibre area, training heart rate and pain measurements and satellite cell counts), the mean of the group was calculated by first calculating the mean for each subject. When data were only reported, standard deviations were used, but when means were compared, standard errors were used. A p-value of less than 0.05 was considered significance.

CHAPTER 5: RESULTS

5.1. HIGH INTENSITY TRAINING

5.1.1 Subjects

All but one subject completed the study. This subject became ill during his period of uphill training and is only included in the baseline data. However, not all subjects completed post-training performance tests. Two subjects did not complete a post-training 10 km time trial - both subjects were in the uphill group; one suffered an ankle sprain and one experienced severe exhaustion (blow-out) during the race. Another subject did not perform post-training laboratory or field performance testing due to a muscle injury (hip flexor muscle) sustained after his last HIT session (in DH group). The subjects that could not complete performance tests were not different to the remaining subjects regarding prior training experience or baseline performance parameters. All subjects except the one that did not complete training were included in the biopsy analysis (n =12).



5.1.2 Training intervention

12 subjects, 6 per group, completed 4 weeks of high intensity training (see Table 5.1 for detail). However, DH subjects completed ~6 intervals per training session despite the frequent increases in training speed. UP subjects struggled to finish 6 intervals in a training session, and only a few subjects could achieve this at the end of the training period. Therefore the DH subjects completed significantly more intervals per session than the UP subjects ($p < 0.01$). UP subjects often reported muscle fatigue and could not recover fully between training sessions. Some DH subjects reported feeling muscle fatigue nearing the end of the 4th week of training.

Subjects in the DH group trained at a higher speed ($p < 0.005$), completed more intervals in total ($p < 0.05$) and correspondingly ran a greater distance during the laboratory training (6.0 ± 0.6 vs. 3.5 ± 0.7 km/session, $p < 0.0005$) than UP subjects. UP subjects exercised at a greater percentage of their maximum heart rate than DH subjects (Table 5.1.2, $p < 0.005$). Subjects' recovery heart rates (measured as the %HR max at the end of the 4th rest interval, see methods) were not significantly different between the two groups (UP: 71.8 ± 1.3; DH: 73.2 ± 5.9; $p = 0.7$). Feelings of fatigue reported immediately after each training session tended to be higher in UP than in DH subjects (Borg's scale of perceived exertion, $p = 0.07$).

Table 5.1 ***High intensity interval training***

	Uphill training	Downhill training	p value ≤
N	6	6	
Number of sessions	10.3 ± 0.5	10.7 ± 1.0	0.7
Intervals / session †	4.5 ± 0.7	5.7 ± 0.3 *	0.01
Speed (km/h) †	15.8 ± 0.7	21.2 ± 0.9 *	0.005
Total number of intervals	47.2 ± 10.2	60.7 ± 6.2 *	0.05
Total distance (km)	35.8 ± 9.3	63.4 ± 5.4 *	0.0001
Maximum heart rate during 4th interval (%max) †	97.1 ± 1.3	91.9 ± 2.8 *	0.005
Borg rating of perceived exertion (9-20) †	17.2 ± 1.9	15.1 ± 1.6	0.07

Values are means ± SD; p value, difference between groups; * minimum significance achieved ($p < 0.05$), † Values are mean of the means for individual subjects over the 10 training sessions

Compared to training in the month preceding the 4 weeks of high intensity training, total training volume during the 4 week HIT period decreased significantly for UP ($p < 0.05$) and DH ($p < 0.05$) groups (Fig. 5.1). Although DH subjects completed a greater distance during HIT than UP subjects ($p < 0.05$), the total training volume and the volume of outdoor training during the 4 week period did not differ significally between groups.

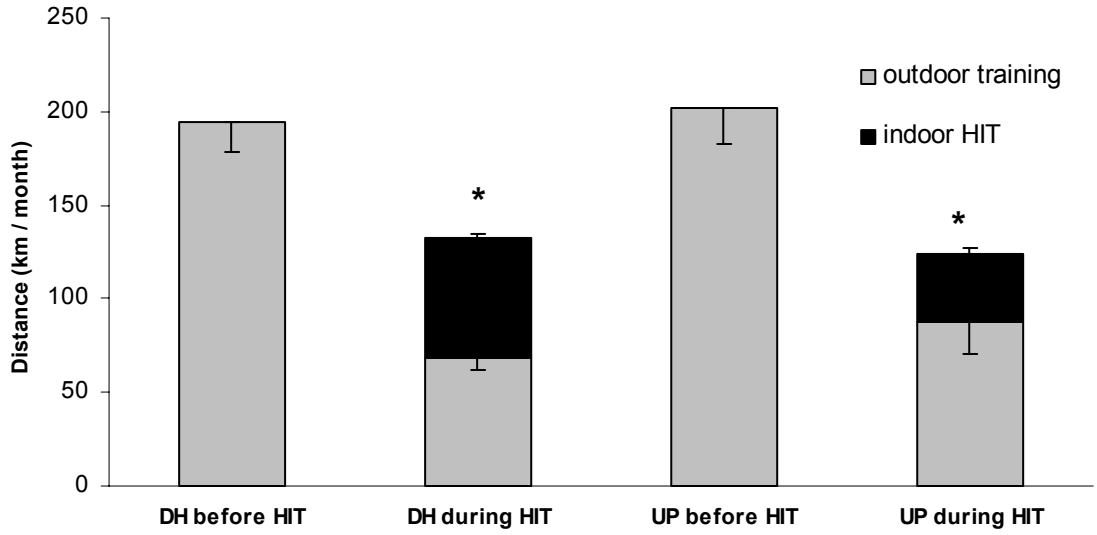


Fig. 5.1 ***Total training volume before and during HIT:***

Values are means ± SE; * Post hoc test, different from before HIT within training group, $p < 0.05$

5.1.3 Perceived muscle pain in response to HIT

Perceived pain after one training session was significantly elevated from baseline only in DH subjects and was higher in DH subjects than UP subjects ($p < 0.005$, Fig. 5.2). This was partly because none of the subjects training uphill experienced sudden or substantial increases in leg pain during the 4 week period. Of the 6 subjects who trained downhill, 3 experienced marked effects of delayed onset muscle soreness (DOMS) after their first training session. This was identified as a substantial increase in pain experienced two days after the exercise bout, as measured by the Visual Analogue Scale (Fig. 5.3). All DH subjects experienced pain associated with delayed onset muscle soreness, but in some subjects this only occurred at a later stage during the 4 week period. Based on pain data, DH training had a greater impact on skeletal musculature, while heart rate data indicate UP training induced a more severe metabolic demand.

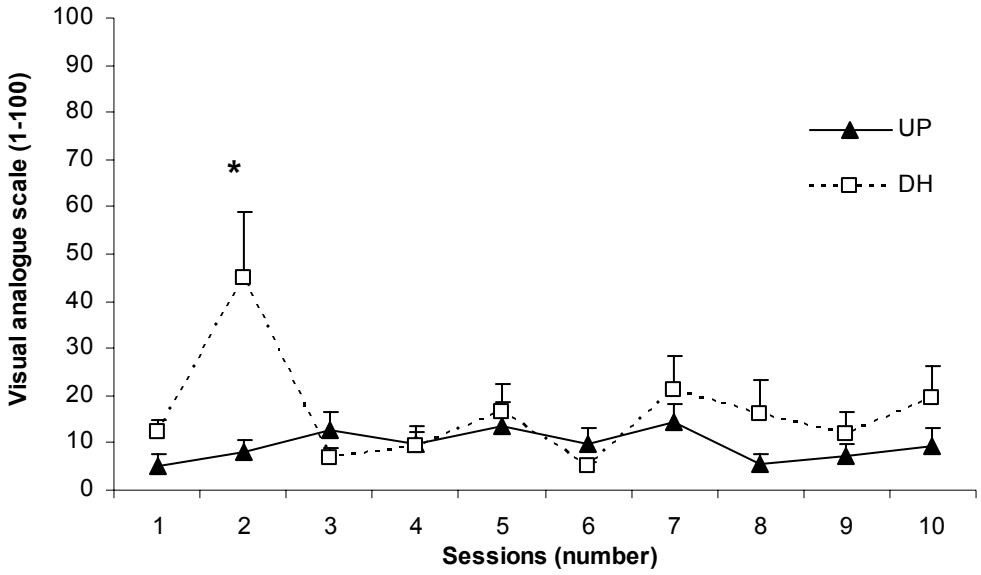


Fig. 5.2 ***Percieved muscle pain in response to HIT:***

Values are mean ± SE; * group *x* time effect, different from baseline & UP at corresponding time point, $p < 0.005$

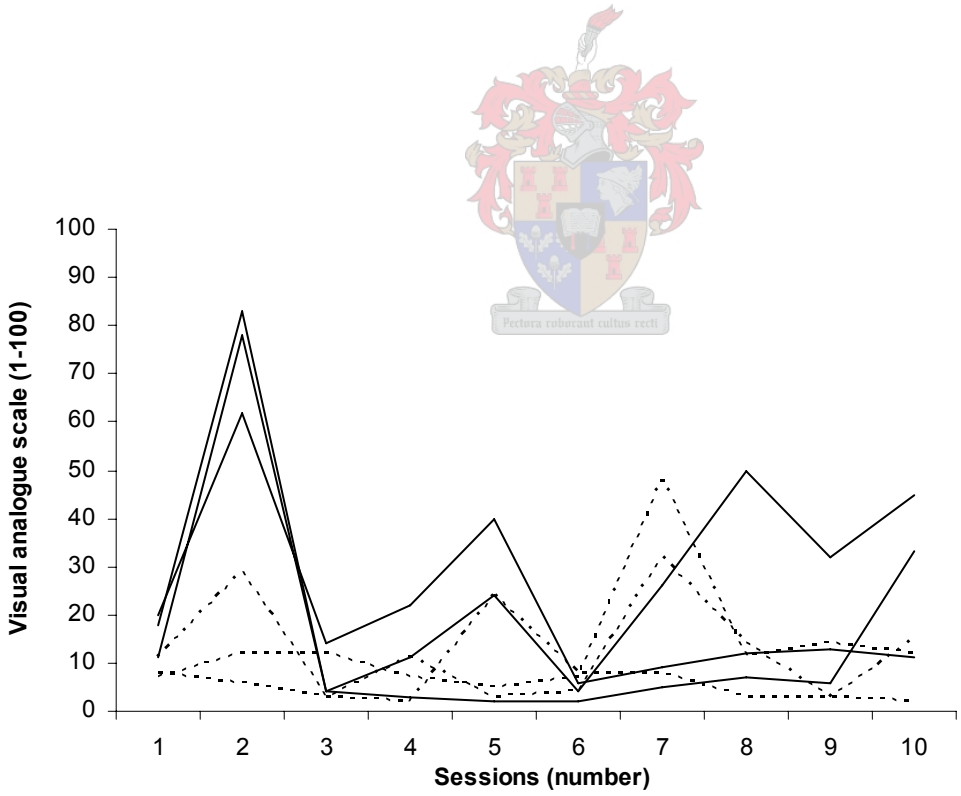


Fig. 5.3 ***Perceived muscle pain: individual DH subject responses***

DOMS, delayed onset muscle soreness

5.1.4 Changes in performance after 4 weeks HIT

The effects of the training intervention on performance were assessed firstly as a main effect of time and if this was significant, the effect of each group was assessed separately (post hoc). After 4 weeks of high intensity training, mean VO_2max achieved during the horizontal and inclined treadmill incremental tests to exhaustion did not change significantly. Similarly, peak treadmill speed (PTS) for the horizontal test did not change significantly.

Table 5.2 ***Changes in performance parameters after 4 weeks HIT***

	Uphill training		**Downhill training**	
	baseline	post-training	baseline	post-training
Laboratory tests				
n	6		5	
VO_2max_{flat} (ml/kg/min)	61.4 ± 1.9	60.6 ± 2.2	61.6 ± 1.5	60.1 ± 2.2
VO_2max_{uphill} (ml/kg/min)	62.1 ± 2.1	64.2 ± 1.0	63.0 ± 0.7	61.3 ± 1.4
PTS_{flat} (km/h)	20.4 ± 0.3	20.4 ± 0.4	20 ± 0.4	20.0 ± 0.4
10 km time trial				
n	4		5	
10 km time (min)	38.23 ± 1.10	38.54 ± 1.14	39.47 ± 0.94	41.09 ± 0.79 *
5 km time - up (min)	19.57 ± 0.46	19.33 ± 0.56	19.98 ± 0.42	20.57 ± 0.49
5 km time - down (min)	18.67 ± 0.63	19.21 ± 0.59	19.49 ± 0.58	20.52 ± 0.34

Values are means ± SE; *n,* no. of subjects; VO_2max, maximum oxygen consumption; PTS, peak treadmill speed; min, time in minutes converted to digital time; statistics, repeated measures ANOVA; * different from baseline, P <0.05;

After HIT training, DH subjects had a worse 10 km running performance compared to baseline (4%, $p < 0.05$, Table 5.2). As two UP subjects could not complete the 10 km time trial, UP results are only for the remaining 4 subjects.

Despite most of the tests indicating that an increase in training intensity which is accompanied by a decrease in training volume does not improve performance, a positive result was obtained in one performance test. There was a significant improvement in their PTS for the inclined treadmill test after training ($p < 0.05$, Fig. 5.4). This was not due to only one group, since post hoc tests did not indicate significance despite the fact that the improvement in the UP group was 4.2% whereas in the DH group it was only 2.4%.

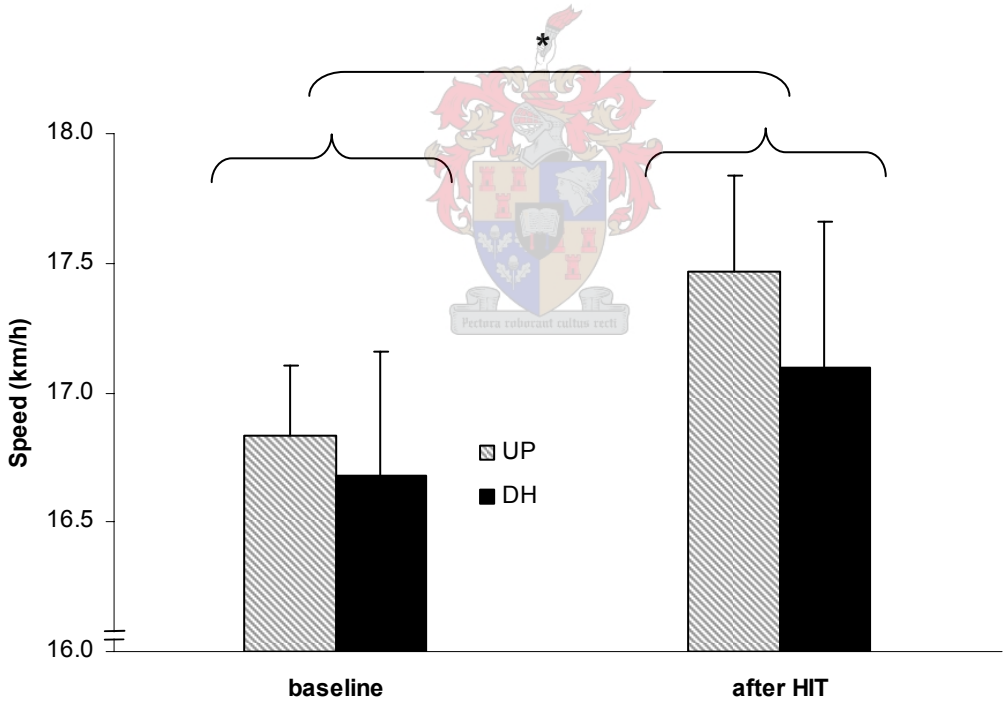


Fig. 5.4 ***Uphill test peak treadmill speed before and after 4 weeks HIT***
Values are mean ± SE; * main time effect for both groups, $p < 0.05$

5.2 MYOGENIN PROTEIN EXPRESSION DURING 4 WEEKS HIT

A repeated measures ANOVA revealed a significant time effect for myogenin expression ($p = 0.006$, Fig. 5.5). Myogenin protein expression tended to increase after 4 weeks of either uphill (84%, 130 ± 18 vs. 239 ± 26, ± SD, units of optical density) or downhill (63%, 160 ± 28 vs. 260 ± 47, ± SD, units of optical density) training, but no post hoc effects were observed in either group. Specific differences between time points did not reach significance due to the high variability of myogenin expression, especially after the first two training sessions (Fig. 5.5). In Fig. 5.6 myogenin is expressed as a ratio of baseline values (=1), providing a view of the trends without the influence of individual variability at baseline. Individual responses in downhill and uphill trained subjects can be seen in Fig. 5.7. After two training sessions, myogenin protein expression was upregulated in some ($n = 2$) downhill trained subjects, but these subjects' myogenin response did not remain elevated at 4 weeks. The other 4 DH subjects' muscle myogenin became elevated at some time after 2 sessions and remained elevated at 4 weeks. In contrast, no UP subjects responded after 2 sessions, but similarly some responded ($n = 4$) with increased expression between 2 sessions and 4 weeks of training.

Early (after 2 sessions) myogenin expression in downhill subjects correlated ($r = 0.985$, $p < 0.005$) with the perceived pain experienced on the same day (Fig. 5.8).

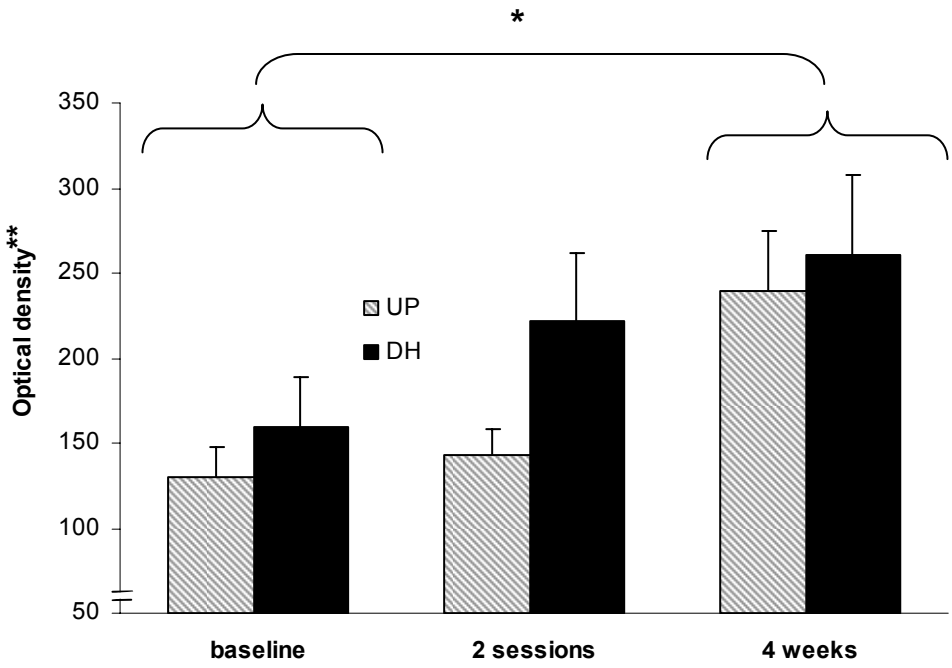


Fig. 5.5 ***Time course of myogenin expression in response to HIT***

Values are mean ± SE; ** optical density expressed relative to one subject's baseline designated a value of 100; * main time effect for both groups, different from baseline, $p < 0.01$

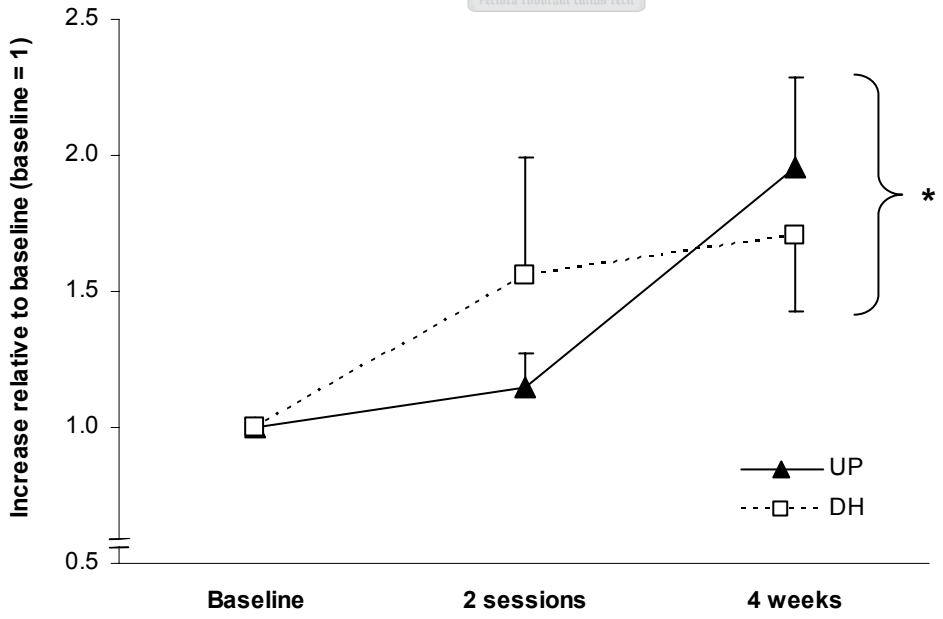


Fig. 5.6 ***Myogenin expression in response to uphill and downhill training***

Values are mean ± SE; * main time effect for both groups, different from baseline, $p < 0.01$; DH, $n = 5$; UP, $n = 6$

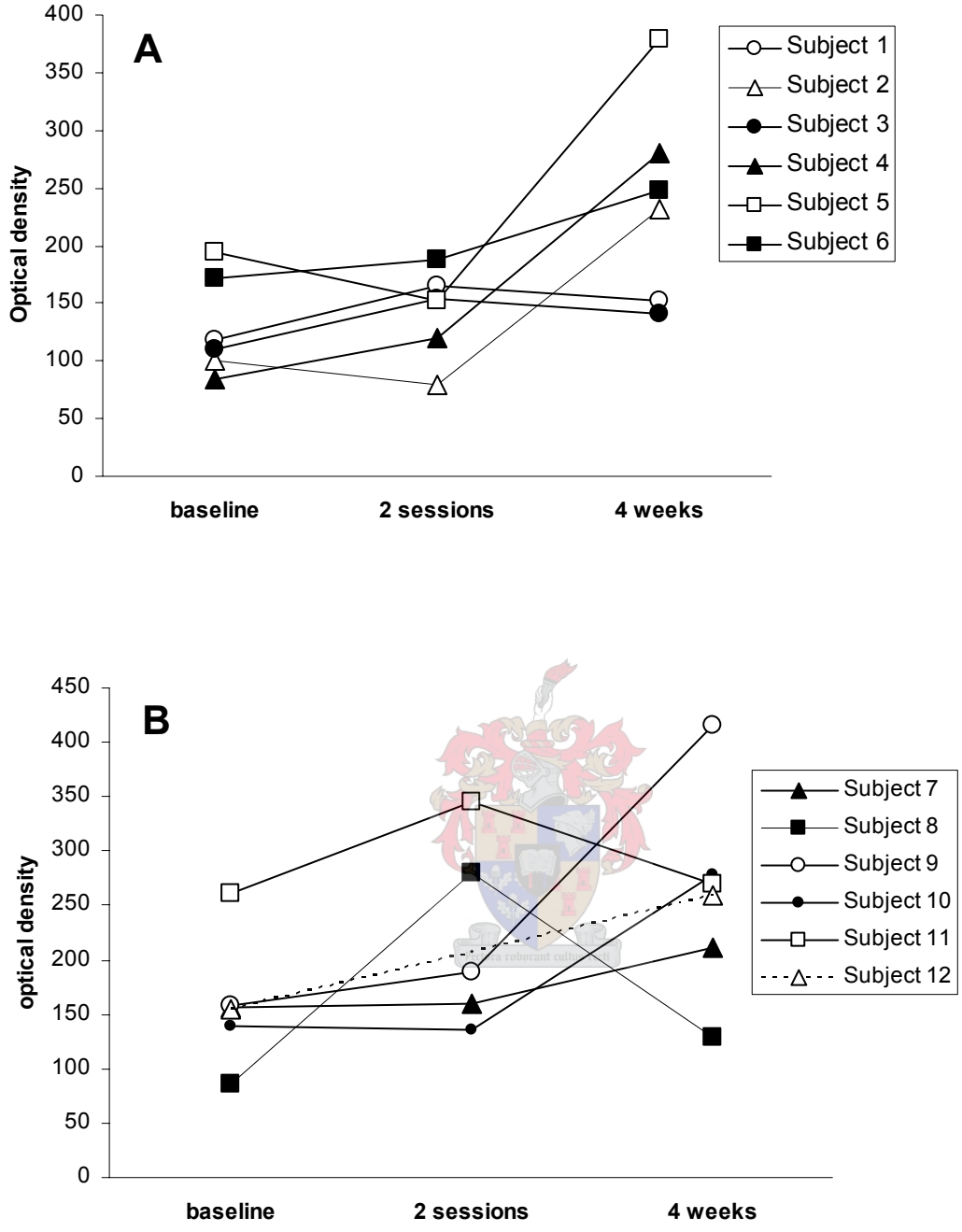


Fig. 5.7 ***Myogenin response to HIT: individual subject responses***

Panel A, UP subjects; Panel B, DH subjects; dotted lines represent data with a missing data point that was not included in statistical analysis

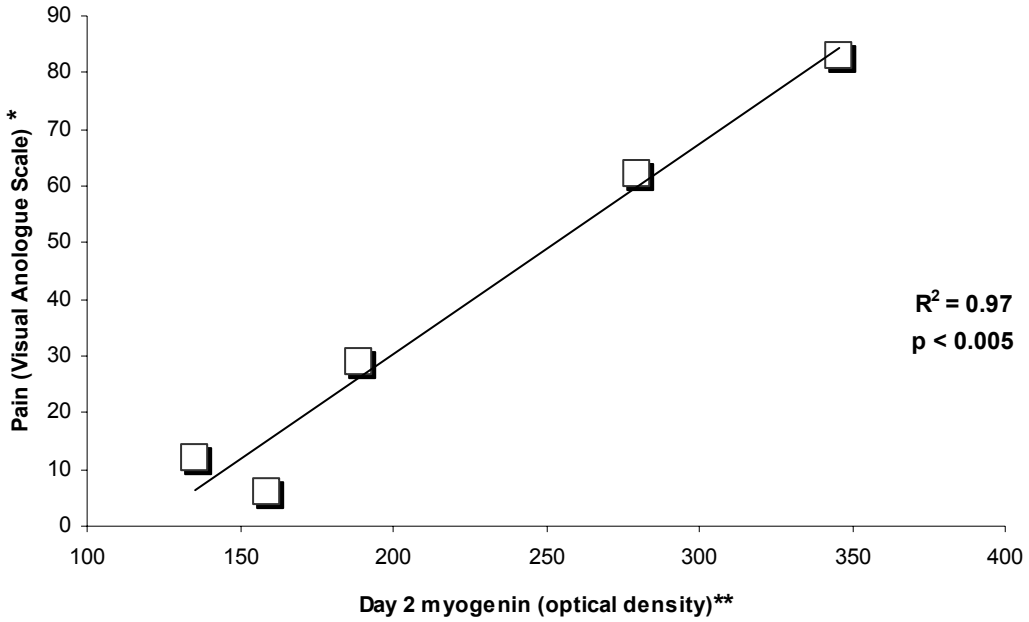


Fig. 5.8 ***The relationship between myogenin expression and perceived pain ~50 hours after the first training session in DH subjects.*** * VAS pain measured before training session 2; ** myogenin protein expression 4 – 6 hr after training session 2

5.3. SATELLITE CELL QUANTIFICATION

CD56 and M-cadherin expression was assessed to quantify the number of satellite cells per muscle fibre, in cross-sections of *vastus lateralis* muscle biopsies.

5.3.1 CD56+ expression per fibre

There was a main effect of time on CD56 expression ($p < 0.05$), and post hoc analysis showed no changes after 2 sessions of training, but a significant increase in CD56 expression between baseline and 4 weeks only in DH subjects ($p < 0.01$). After 4 weeks of training, CD56+ cells per fibre increase noticeably in DH subjects compared to baseline (138%, $p < 0.05$) but not significantly in UP subjects (39%, NS). For the number of CD56 positive stained cells, there was no significant difference between UP and DH subjects at

any time point, even at 4 weeks, indicating a trend for elevation even in UP subjects (Fig. 5.9). Nonetheless, individual subject variation is illustrated in Fig. 5.10. These graphs indicate that *n* = 2 subjects in the UP group were responders by 4 weeks. I have designated a responder as being a subject who increased CD56 expression by > 0.25 CD56 cells / fibre. Five out of six DH subjects were responders at 4 weeks.

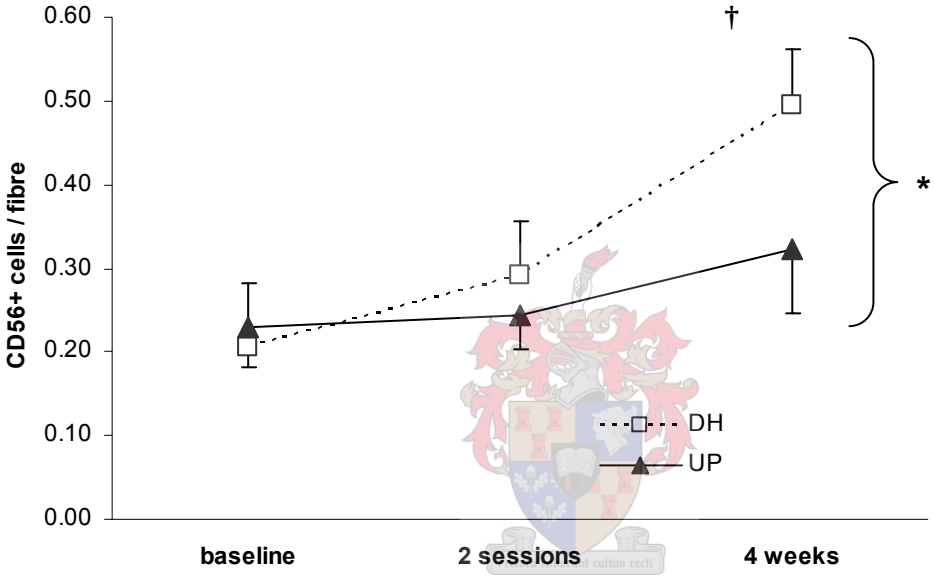
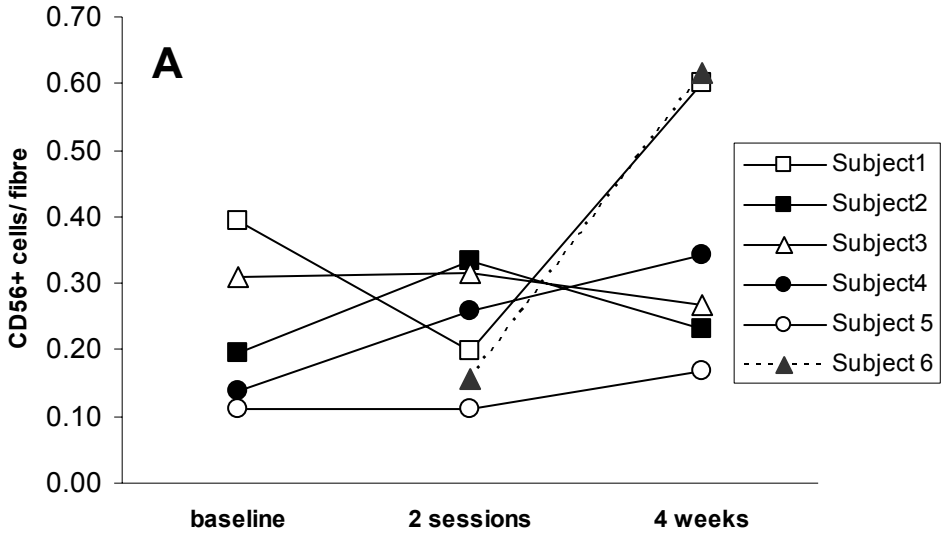


Fig. 5.9 ***Change in CD56+ cells / fibre during HIT***

Values are means ± SE; DH, *n = 4* ; UP, *n* = 5; * main time effect for both groups, different from baseline, p < 0.05, time effect ; † post hoc difference from baseline within group, p < 0.01



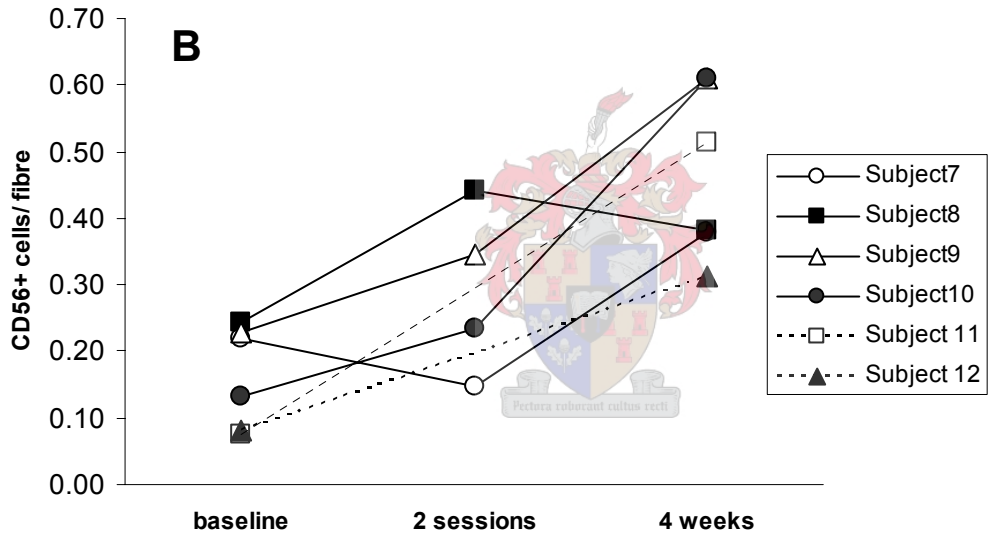


Fig. 5.10 ***CD56+ cells / fibre : Individual subject responses***

A, UP subjects ; B, DH subjects; dotted lines represent data with a missing data point that were not included in the previous figure or in statistical analysis

5.3.2. M-cadherin⁺ expression per fibre

Mcad+ expression followed a similar trend to CD56⁺ expression. There was no time-effect after 2 training sessions. However after 4 weeks a significant main effect of time, different from baseline, was observed ($p < 0.05$). After 4 weeks of training, Mcad⁺ cells per fibre

increased considerably in DH subjects (123%, $p < 0.05$) and not significantly in UP subjects (45%, NS) compared to baseline. No differences were found after 2 sessions or between training groups at any time point (Fig. 5.11).

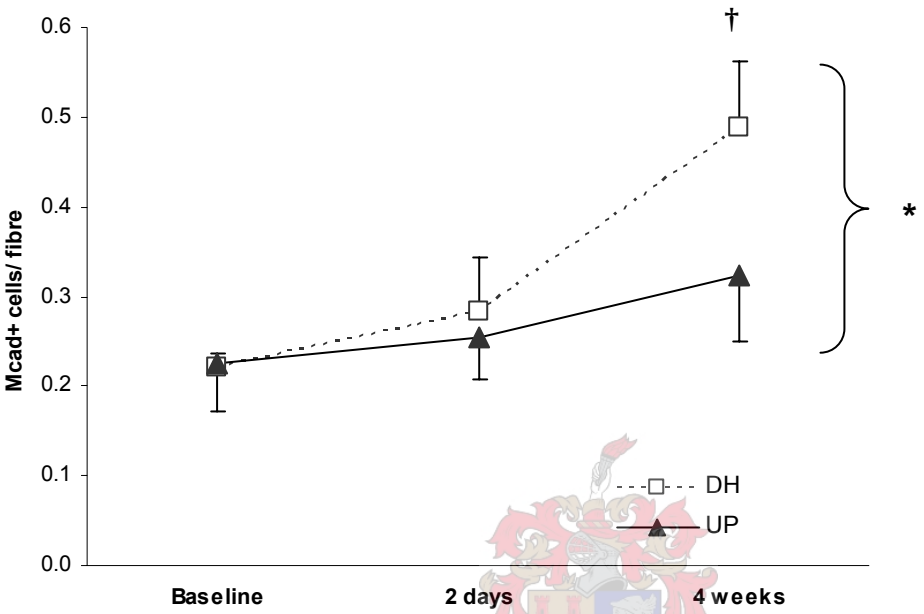


Fig. 5.11 ***M-Cadherin expression / fibre in response to HIT***

Values are means ± SE; DH, *n* = *4* ; UP, *n* = 5; * main time effect for both groups, different from baseline, $p < 0.05$, time effect ; † post hoc difference from baseline within group, $p < 0.01$

5.3.3 Additional immunohistochemical data

In the preceding data presentation, the number of satellite cells was expressed per fibre. But there are other possible ways to express these data. The first is to determine the number of satellite cells relative to the surface area of muscle analysed and the second relative to myonuclei identified as either satellite cell nuclei or muscle cell nuclei. Satellite cell frequency expressed relative to surface area, as well as satellite cells per total myonuclear content, followed similar trends to those presented in detail earlier for satellite cells expressed per myofibre. These data are presented in Table 5.3.

Table 5.3 ***Additional immunohistochemical data***

	Group	Baseline	2 sessions	4 weeks	Main time effect *
CD56+ cells / 93 000 um^2	DH	3.1 ± 0.4	5.3 ± 0.9	7.9 ± 0.9 †	p < 0.005
	UP	3.4 ± 0.5	3.9 ± 0.6	5.8 ± 1.2	
Mcad+ cells / 93 000 um^2	DH	3.2 ± 0.5	5.1 ± 0.8	7.7 ± 1.0 †	p < 0.005
	UP	2.9 ± 0.6	4.0 ± 0.8	5.9 ± 1.3	
CD56+ cells / nucleus (%)	DH	6.5 ± 1.1	10.4 ± 2.6	15.2 ± 2.0 #	p < 0.01
	UP	6.9 ± 0.8	9.9 ± 1.4	10.9 ± 1.3	
Mcad+ cells / nucleus (%)	DH	6.7 ± 0.9	10.1 ± 2.4	15.0 ± 2.2 †	p < 0.005
	UP	7.0 ± 1.2	9.3 ± 0.8	10.9 ± 1.3	

values are mean ± SE ; * overall time effect for both groups p < 0.05 ; † post hoc test, different from baseline within group, p < 0.05 ; # post hoc test, tendency to be different from baseline within group, p = 0.07

5.3.4. M-cadherin vs. CD56 staining

Mcad and CD56 expression were similar at all time points in both groups. Therefore I pooled the groups for the following analysis. A strong correlation was found between Mcad and CD56 expression over all three time points (Fig. 5.12; r = 0.95, p < 0.0001). Within time points, the correlations were also evident: baseline, r = 0.89 ; 2 days, r = 0.87 ; 4 weeks, r = 0.97 (all p < 0.0001).

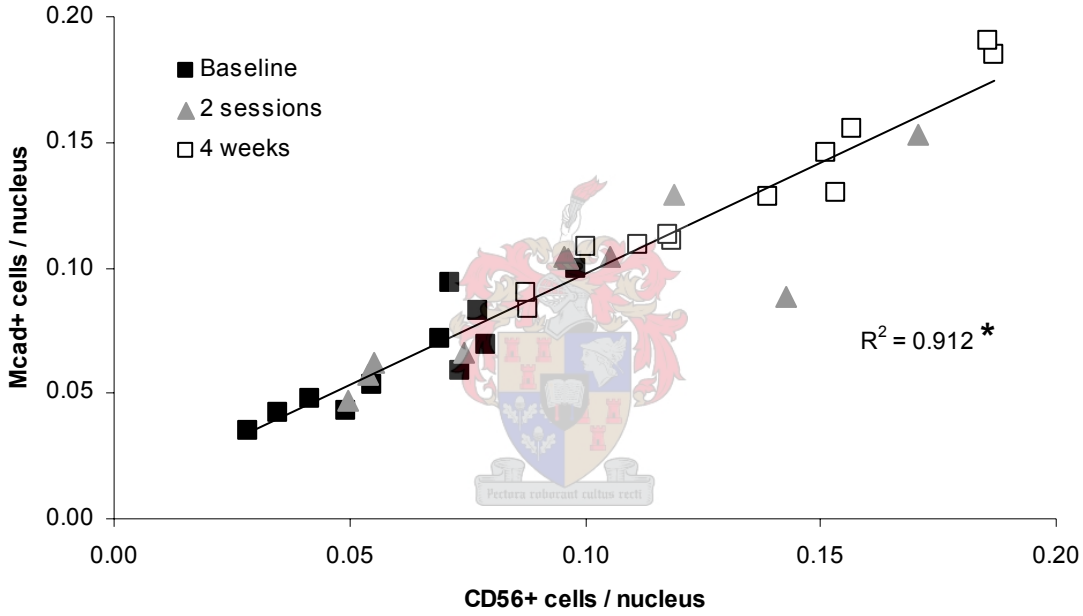


Fig. 5.12 ***CD56+ vs. M-cadherin+ staining***

Values = mean for each biopsy sample analysed, * r = 0.95, p < 0.0001

5.4. FIBRE CROSS-SECTIONAL AREA

Fibre area did not change after 4 weeks of HIT in DH or UP subjects (Fig. 5.13). However, a statistical comparison was done to compare the sizes of the standard deviations at baseline versus after 4 weeks. Significantly smaller standard deviations of the mean were seen after 4 weeks HIT ($p < 0.05$). Statistics were done as described by Morrison (Morrison, 1978). The histograms in Fig 5.14 illustrate the 'shift' towards a more uniform fibre area.

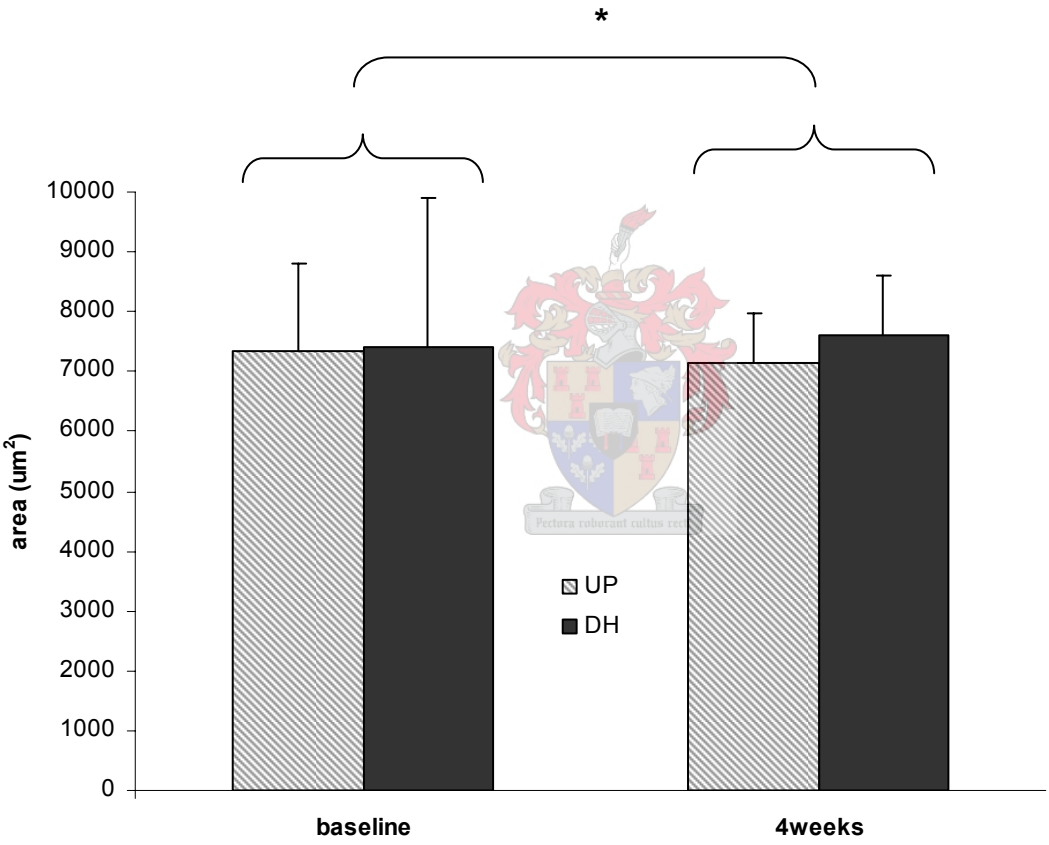
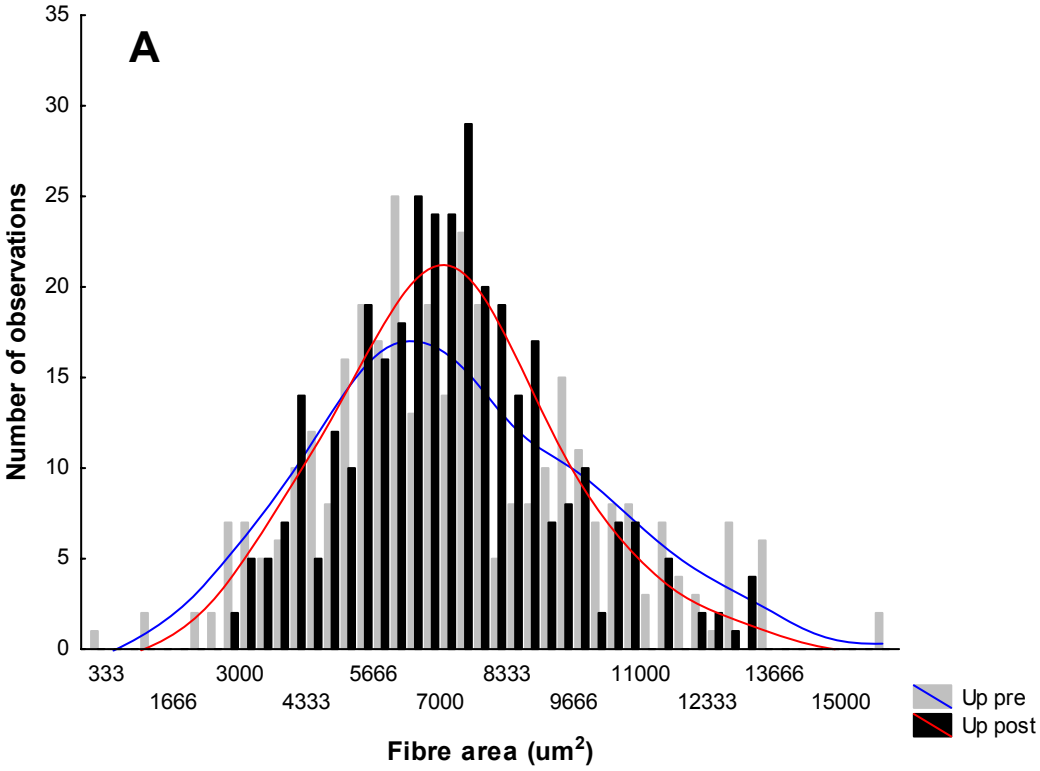


Fig. 5.13. ***Mean fibre cross-sectional area before and after 4 weeks HIT***

Values are means ± SD ; * overall time effect for both groups, SD different from baseline, $p < 0.05$, analysed according to Morrison (1978)



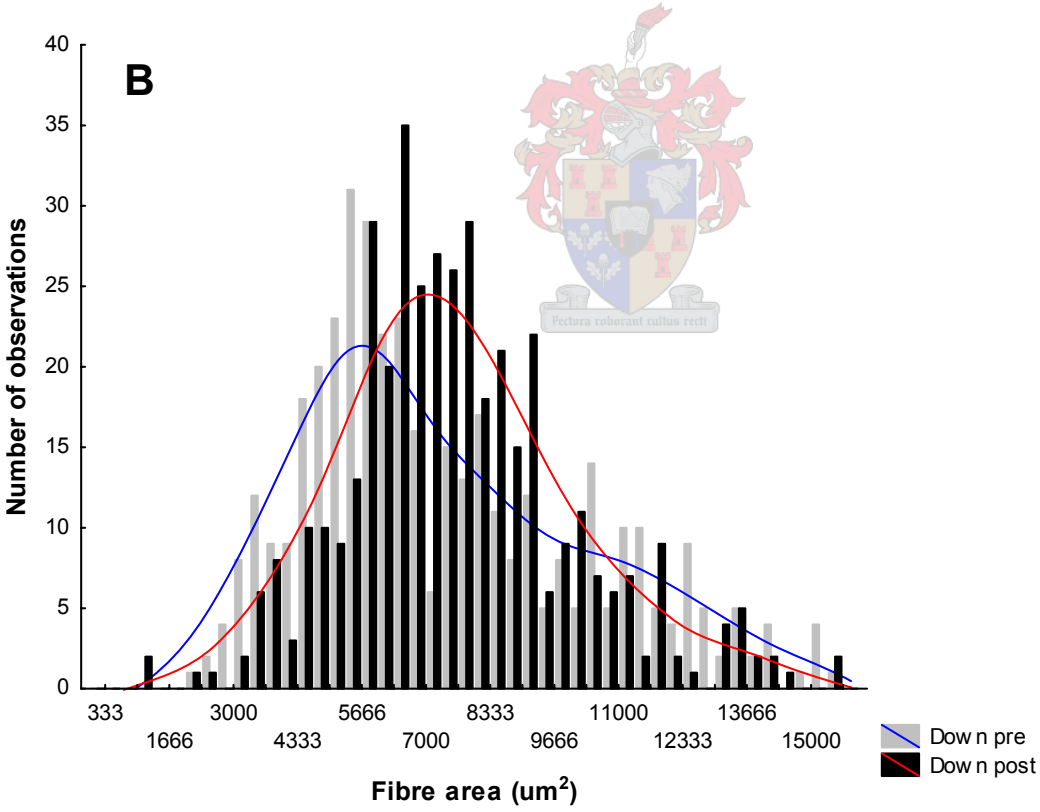


Fig 5.14 ***Mean fibre cross-sectional distribution before and after 4 weeks HIT***
Panel A, UP subjects, *n* = 5; Panel B, DH subjects, *n* = 6; a total of 70 fibres are included for each subject.

5.5. MYOSIN HEAVY CHAIN COMPOSITION

Myosin heavy chain composition did not change significantly over time in either group during 4 weeks of training (Fig. 5.15). ANOVA indicated a main effect of group for type IIx fibres with DH having significantly greater percentage MyHC type IIx than UP ($p < 0.05$).

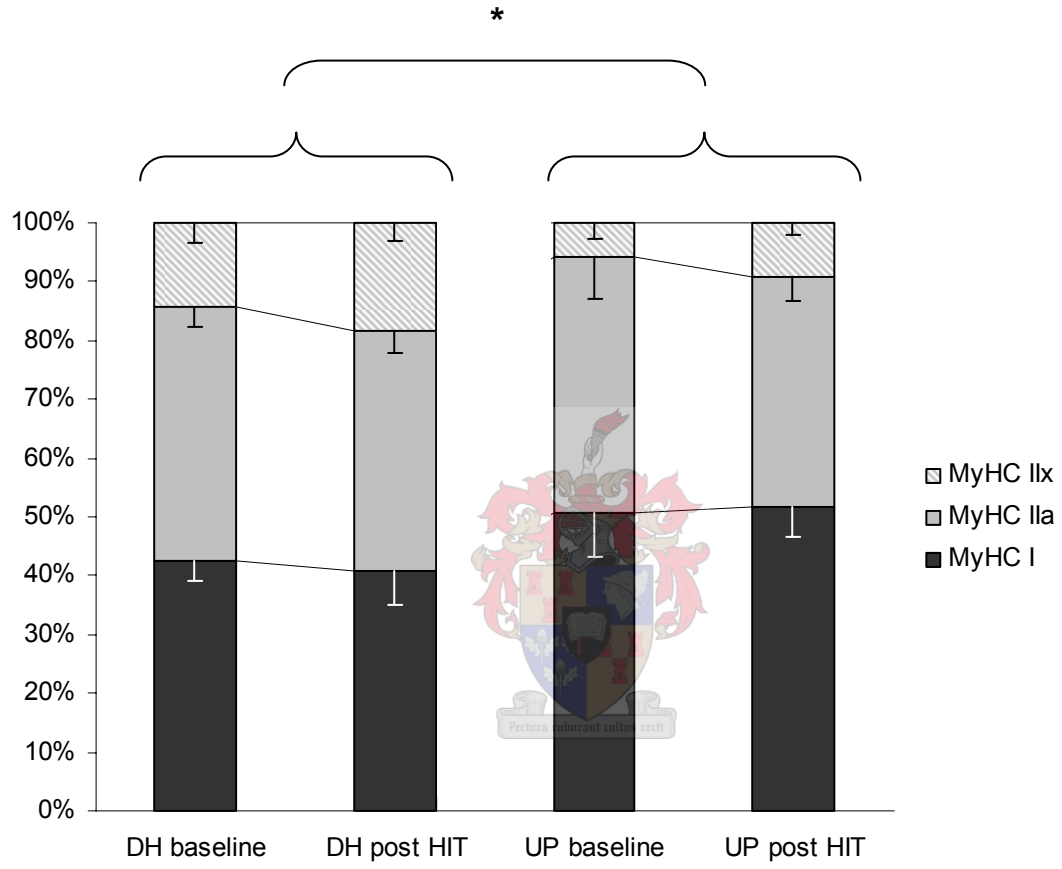
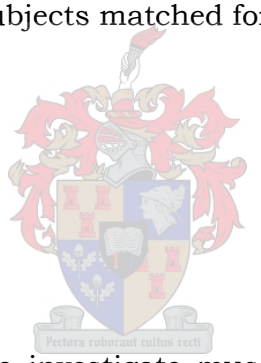


Fig. 5.15 ***Myosin heavy chain distribution before and after 4 weeks of HIT***

Values are means ± SE**;** * overall group effect, $p < 0.05$, for MyHC IIx

CHAPTER 6: DISCUSSION

Few studies in the past have followed the time course of satellite cell proliferation in human subjects in response to exercise training irrespective of training mode (Charifi *et al.*, 2003;Crameri *et al.*, 2004;Kadi *et al.*, 2004b). Although short term responses to DH training have been investigated (Byrnes *et al.,* 1985; Malm *et al.,* 2004; Smith *et al.,* 1998) to my knowledge, no study has investigated satellite cell proliferation in response to a training intervention lasting at least 4 weeks, in a group of already-trained runners. This study is the first to report changes in satellite cell proliferation and the myogenic regulatory factor, myogenin, in runners in response to a change in training, specifically chronic high intensity endurance training (HIT). I have investigated two types of HIT, up- and downhill interval running, in subjects matched for previous training volume.



6.1 STUDY DESIGN

The main aim of this study was to investigate muscle adaptation to chronic downhill running. To induce a sufficient downhill stimulus in trained endurance runners familiar with road running, I used a high intensity intervention. Therefore, there were actually two interventions at the same time, an increase in downhill running and higher intensity of training. Therefore, choosing an appropriate control group was difficult. Ideally the control group should also perform HIT, so that the response to downhill running can be assessed separately from the response to HIT. Because HIT on a flat surface still contains a large proportion of eccentric work, an uphill high intensity running group would present a better control. Matching of the subjects, who were divided into downhill and uphill high intensity interval running groups, was confirmed by similar performance for both groups in incremental exercise tests to exhaustion at baseline.

Other studies have assessed the response to intermittent DH running after one (Malm *et al.*, 2004;Peake *et al.*, 2005) or two sessions (Byrnes *et al.*, 1985;Smith *et al.*, 1998). I designed a downhill training programmeme that was continuously adjusted to provide an ongoing stimulus to provoke muscular adaptation. Average interval speed increased from 19.0 ± 0.9 km/h (mean ± SD) in session 1 to 22.6 ± 1.0 km/h by session 10. Previous studies used 45 min of downhill running at the intensity of 50-70% VO_2max to elicit a DOMS response. In contrast, I used only 23 min of intermittent running (6 intervals of 3 min, 1 min rest intervals), but the intensity of training was far greater. Subjects in this study trained at speeds well in excess of 20km/h, compared to an average speed of 12 – 14 km/h in previous studies. This difference in speed is partly due to the better running ability of subjects in the present study, but also due to the objective of this study requiring a higher running intensity (95% PTS). While other researchers' only aim was to induce DOMS, I investigated the adaptation to chronic and progressive downhill training.

Previously, Lastayo *et al.* matched the intensity of eccentric and concentric cycling training according to heart rate (LaStayo *et al.*, 2000) and VO_2 (LaStayo *et al.*, 1999). He could easily achieve this, as training was done at sub-maximal exercise intensity (~60% HRmax). I also attempted to match uphill and downhill training for training intensity (% HRmax), but did not achieve this, as UP subjects exercised at a significantly higher percentage of their maximal heart rate (92 vs. 97% HRmax). However, since UP speed was already similar to subjects' 10 km race pace, a further reduction in training speed could have been counter-productive. Although DH subjects exercised at a lower percentage of their maximum heart rate, training intervals were longer and the number of intervals more than those for UP training subjects. Therefore, the HIT stimulus was still similar between groups, especially since ratings of perceived exertion were not significantly different between the two groups. Therefore, all UP subjects performed a concentrically biased training intervention as a control for the eccentrically biased downhill running experimental group. Still, direct comparisons between the two training

groups should take into account that in both groups training intensity increased. This allowed us to assess changes due to increased training intensity (both groups) versus downhill running (only one group).

I used leg pain assessments prior to each interval session to determine, subjectively, the possible effectiveness of the eccentric protocol for eliciting skeletal muscle adaptation. Reported leg pain was extremely variable between DH subjects, with some subjects experiencing delayed onset muscle soreness (DOMS) after one training session, whilst others did not. Other studies that have elicited DOMS after one session typically use untrained or less trained subjects (Byrnes *et al.*, 1985;Cleary *et al.*, 2002) or more severe protocols isolating a single muscle (Cleak & Eston, 1992;Newham *et al.*, 1987). However, my aim was to use a protocol that could be applied to athletes as a training stimulus, rather than with the sole purpose of experimentally instigating muscle microdamage. The initial speed of DH training (95% PTS_{flat}) as well as the duration of training (6 x 3 min) may not have been enough of a stimulus to trigger DOMS in some subjects. The extent to which subjects included undulating routes into their prior training may have had an influence on their responses to DH running due to the known 'repeated bout' adaptive effect (Byrnes *et al.*, 1985). Subjects differed within each group for running performance, training volume and months of regular endurance training. However, there was no correlation between leg pain after the 1st session and either running performance ($r = 0.62$, $p = 0.20$) or training volume ($r = 0.53$, $p = 0.28$). Months of prior endurance training experience was not quantified, but noticeably the most experienced runner in the downhill group did not experience any DOMS after the first training session, while the subject with the shortest running history, experienced severe DOMS. It seems that multiple sessions of downhill training may have a cumulative effect until a certain 'threshold' is reached, as subjects who did not experience DOMS after their first training session, experienced muscle pain at a later stage during the training period (after 4-6 sessions). This may indicate an accumulation of microdamage until a certain amount of

eccentric stress has been endured. Alternatively, since the speed of the intervals increased progressively, it may merely be that a 'threshold' speed, specific to each subject, had been reached (relative to initial training speed). This theory is purely speculative as I do not have evidence to substantiate it. However, further investigations of these two possible explanations may be vital to understanding the response to repeated eccentric exercise bouts.

6.2 SATELLITE CELL PROLIFERATION

Baseline satellite cell frequency

This study is the first to report data on the satellite cell frequency of young endurance trained runners, and thus it is difficult to evaluate relative to comparable previous research. However, my data compares well with several studies on healthy males. Roth *et al.* (2001) reported that ~2.5 % of myonuclei were satellite cells in *vastus lateralis* muscle of young men. Similarly, Crameri *et al.* (2004) reported a satellite cell frequency in *vastus lateralis* muscle of 2.59 ± 1.05 % satellite cells per total myonuclei in sedentary males. In the same study, levels were elevated to 6.38 ± 1.23 % two days after a bout of high intensity eccentric resistance training. Erriksson *et al.* (2005) reported 9.4 ± 3.0 % satellite cells per total nuclei in the *vastus lateralis* muscles of professional power lifters. In the present study, baseline satellite cell frequency was 6.2 ± 2.1%, which falls between values reported for sedentary men and professional power lifters. In another recent study, healthy men ranged from 1.9 ± 0.7 % to 2.5 ± 0.8 % satellite cells per total myonuclei (*vastus lateralis* muscle). This value escalated to 15 ± 1.6 % after 20 weeks of testosterone supplementation (Sinha-Hikim *et al.*, 2003). Therefore, satellite cell frequency seems to be responsive to a variety of stimuli, including acute exercise, chronic exercise and hormonal environment.

When my results are compared according to satellite cells per fibre, they also compare well with previous work. Kadi *et al.* (2004b) reported ~0.14 satellite cells per fibre (assessed in cross-sectional orientation) in healthy subjects and the frequency increased to 0.18 after 3 months of resistance training. In my study, endurance trained subjects had 0.19 ± 0.10 satellite cells per fibre at baseline. Therefore, their satellite cell frequency is that expected for young men participating in regular training.

I used both CD56 and M-cadherin to identify satellite cells. Earlier studies have used one marker (or method), but comparison of results show that studies using m-cadherin (Sajko *et al.*, 2004), CD56 (Crameri *et al.*, 2004) and electron microscopy (Roth *et al.*, 2001) have found similar satellite cell frequencies for young men. The two proteins have previously been shown to be co-expressed frequently during muscle development (Rose *et al.*, 1994) and in denervated muscle (Irintchev *et al.*, 1994) in mouse models. However, another study observed partial co-expression of m-cadherin and CD56, but also a higher frequency of CD56+ nuclei than m-cadherin+ nuclei in rat muscle irrespective of denervation or age (Dedkov *et al.*, 2003). I found a very strong correlation (r = 0.95) between CD56 and M-cadherin staining across all time points. This study is therefore the first to show consistent co-expression of these two antibodies in adult human muscle. Whether this finding is specific to trained athletes, or whether such close concord also exists in sedentary subjects remains to be investigated.

Early changes in satellite cell frequency in response to HIT

In rat studies, satellite cell proliferation did not occur within 24 hours after treadmill running (Darr & Schultz, 1987;Jacobs *et al.*, 1995;Smith *et al.*, 2001). Therefore, the biopsies taken 4-6 hours after the 2nd training session are likely to reflect influences on the satellite cell population in response to the 1st HIT session (early) and the biopsies taken 4-6 hours after the last training session reflect the accumulation of responses to all but the last session.

In the present study, satellite cell frequency was increased insignificantly, by 50%, 2 days after the first DH exercise session. Previous research has shown dramatic increases in satellite cell frequency in the days following an acute bout of unaccustomed eccentric training which ranged from eccentric downhill running in rats (Darr & Schultz, 1987), to eccentric resistance training in humans (Crameri *et al.*, 2004). Similar to the present study, Crameri *et al.* observed a substantial, yet insignificant, increase in satellite cell frequency 2 days after eccentric exercise in sedentary men: satellite cell proliferation was increased by 145% at two days, but reached peak values at 4 days post-exercise, when they were elevated ~200% above baseline (Crameri *et al.*, 2004). This implies that 2 days post exercise in the present study was possibly too early to detect maximal satellite cell proliferation in response to the 1st session.

A second reason for the lack of significant change within 2 days after exercise was the large individual variation seen within both training groups. Although I matched subjects according to their training volume and running performance so that the means of the two groups did not differ significantly, variation within the groups in these factors was present. Possibly other individual differences were also present. Factors such as training experience, cross-training with others sports or even inherent resistance to exercise-induced muscle soreness, could have influenced a subject's individual response to HIT. Another possible confounding factor may be the variability of satellite cell frequency in baseline samples. Although subjects were 'rested' at the time of baseline biopsies, they were already in a trained state due to regular training. It is possible, and highly probable, that some subjects were training more strenuously in the month preceding the training intervention, leading to elevated levels of satellite cells at the time of baseline testing. Notably, the two subjects with the highest baseline satellite cell frequencies (> 0.3 SC/fibre), only started endurance training on a systematic basis fairly recently (<6 months) and therefore were not fully conditioned to the demands of regular training. Importantly, these two subjects showed no increase in satellite cell frequency (in one a

decrease) in response to the first acute HIT bout, although both were in the UP group. For subjects with intermediate satellite frequencies (> 0.15; < 0.30 SC/fibre) at baseline, no particular early response pattern existed, implying that multiple factors add to the complexity of early satellite cell proliferation in response to HIT. Despite intra-subject variability it is evident that DH subjects tended to respond earlier to HIT (3 of 4 subjects) while in UP subjects, satellite proliferation was only seen at a later stage and only clearly in a few subjects (*n*=2).

Changes in satellite cell frequency after 4 weeks of HIT

One of the main aims of this study was to investigate satellite cell proliferation in response to chronic uphill and downhill high intensity training. Although no statistically significant differences were found between the two training groups, satellite cell proliferation appeared greater in DH than in UP subjects: in UP subjects, satellite cell proliferation was clearly not significantly influenced by 4 weeks of training (CD56/fibre, +39%; MCad/fibre, +45%), while in DH subjects, increases from baseline were significant over time (CD56/fibre, +138%, MCad, +123%, both $p < 0.05$). The extent of satellite cell proliferation seen in DH subjects is far greater than that observed after others types of exercise. Kadi *et al.* (2004b) observed a 31% increase in satellite frequency after 3 months of resistance training in young healthy males. Charifi *et al.* (2003) noted a 29% increase in satellite cell frequency after 14 weeks of endurance cycling in elderly males. These values (31, 29%) are similar to values seen for UP subjects in the present study (39%). However, these studies are difficult to compare as Charifi *et al.* (2003) tested active 70 – 80 year old males, who might be expected to have a reduced myogenic capacity, while Kadi *et al.* (2004b) trained young sedentary males. Importantly, these two mentioned studies, utilized mainly concentric contractions, which is similar to the UP HIT in the present study.

The larger increases in satellite cell number in DH subjects were therefore not due to HIT, but to the eccentric nature of the exercise. Eccentric resistance training (Hortobagyi

et al., 1996a;Hortobagyi *et al.*, 2000;Komi & Buskirk, 1972) and eccentric cycle ergometry (LaStayo *et al.*, 2000) have been shown to be effective at inducing muscle hypertrophy. In one of the few studies to examine chronic eccentric ergometry, authors speculated that the presence of small muscle fibres may be a sign of hyperplasia (Friden *et al.*, 1983a). Although no human studies to date have studied myogenic responses to chronic eccentric exercise, Crameri *et al.* (2004) have shown a 193% increase in satellite cell number 4 days after a single high intensity eccentric resistance bout. My study is the first to show a sustained increase in satellite cell frequency in response to a chronic eccentrically biased training intervention. The reason for the large increase in satellite cell number is still somewhat unclear. Four possible options will be discussed below, namely *i)* hypertrophy, *ii)* neofibre formation, *iii)* shift of fibre type, *iv)* muscle damage

i) In several studies, increases in satellite cell number have coincided with muscle hypertrophy (Charifi *et al.*, 2003;Hikida *et al.*, 2000;Kadi *et al.*, 2004b;Kadi & Thornell, 2000). Satellite cells are thought to proliferate and differentiate in order to be incorporated into enlarging muscle fibres to provide more nuclei. Even in the absence of an exercise intervention, 20 weeks of testosterone supplementation (300mg / week) increased satellite cell frequency by 100% in healthy males. Similar to the aforementioned studies, this change occurred concurrently with increases in fibre cross-sectional area (+32 %) and in myonuclear number (+44%) (Sinha-Hikim *et al.*, 2002;Sinha-Hikim *et al.*, 2003). However, in my study there was no increase in mean fibre cross-sectional area.

ii) Satellite cell proliferation has been shown during endurance training where hypertrophy is not expected. In two rat studies, satellite cell frequencies were greatly increased (2 – 2.5 fold) after 6 – 10 weeks of flat treadmill running (McCormick & Thomas, 1992;Umnova & Seene, 1991). In the one study, no increase in myonuclear density was seen, although small fibres with significantly higher nuclear densities were

observed (McCormick & Thomas, 1992). This may allude to neofibre formation. Indeed, two studies in human subjects have shown evidence of new fibre formation after endurance exercise. Appell *et al.* (1988) have shown the presence of new myotubes as well as activated satellite cells after 6 weeks of aerobic bicycling. In agreement with this finding, Kadi *et al.* (2004a) observed myotubes and small fibres stained positive for developmental MyHC isoforms after 10 weeks of endurance training. They also concluded that this was evidence of new fibre formation.

From the data presented in this section, it seems plausible that satellite cells can be activated for reasons other than muscle damage or hypertrophy, namely the formation of new fibres and an increase in myonuclear density. Although no significant changes in myonuclear number were seen in the present study, it is possible that muscle adapted to exercise through neofibre formation. Unfortunately I cannot be certain of this, as I did not measure developmental MyHC isoforms. I searched muscle sections for evidence of myogenesis and found a few slides that contained dense staining patterns, possibly evidence of new small fibres and myotubes (examples in Appendix).

iii) However, in another animal study, low frequency muscle stimulation was used to induce a shift to a slower muscle phenotype. The shift in fibre type was preceded by a large increase in satellite cell frequency (3.7 fold) as well as an increase in myonuclear number. Although no muscle damage was measured in the study quoted here, developmental MyHC isoforms were observed. No increases in fibre area were measured and new myonuclei were thought to increase the myonuclear density in type IIb cells before conversion to type IIa and I isoforms (Putman *et al.*, 1999). In my study I found no significant fibre type conversion, although it is possible that the duration of the intervention was not long enough.

iv) Although chronic low frequency stimulation does not cause muscle damage in the rat model (Delp & Pette, 1994;Putman *et al.*, 1999;Putman *et al.*, 2000), this intervention is

known to cause muscle damage in the rabbit model (Maier *et al.*, 1986;Schuler & Pette, 1996). In these rabbit studies, muscle degeneration and regeneration were seen as the main stimuli for satellite cell proliferation. Therefore, I cannot ignore muscle damage as a potential stimulus for satellite cell proliferation in the present study. Exercise-induced muscle damage is frequently seen in humans in response to unaccustomed exercise, and especially eccentric contractions (Clarkson & Hubal, 2002). The present study did not focus on markers of muscle damage or inflammation, thus it is difficult to assess the role muscle damage may have played in the training model. However, if I make the assumption that the decrease in exercise-induced muscle soreness towards the end of the 4 weeks of training reflected a reduction in new muscle damage then satellite cell proliferation after chronic downhill running is not exclusively stimulated by muscle damage. But, the proliferative response evident at 4 weeks could still be reflecting the early microdamage.

The absence of changes in myonuclear number in the present study may have several possible explanations. It is possible that, although satellite cells had proliferated and showed evidence of differentiating after 4 weeks of training (see section 6.3 below), they had not been incorporated into myofibres. This is very likely, as increased satellite cell numbers did not lead to increased myonuclear number after 3 months or 14 weeks of training in previous studies (Charifi *et al.*, 2003;Kadi *et al.*, 2004b). During satellite cell proliferation various signals including MRFs, MGF and LIF are responsible for regulating the satellite cell population. Other factors such as HGF, FGF, IL-6 and IGF-1 have more complex actions during myogenesis, including influencing the migration of satellite cells and their fusion to adult myofibres. It is possible that these migration-specific signals were absent or not 'sufficiently' expressed in the present study. However, as I did not measure these signals in this study, I can only speculate on their role. The only myogenic factor measured in this study, myogenin, is associated with satellite cell differentiation, and may provide additional information on the myogenic response.

6.3 MYOGENIN PROTEIN EXPRESSION

Early changes in myogenin protein expression in response to HIT

Early responses to the HIT interventions were measured after subjects' second training sessions. Biopsies were not taken directly after the first training session, since the aim was to first allow for the possible development of DOMS. This protocol could be questioned, as this biopsy may reflect responses to the first or the second training session, or even a combined effect of the two. However, the timing of the biopsy did coincide with the highest reported VAS in the DH group.

Previous research protocols and results varied considerably regarding the early time course of myogenin upregulation after exercise. In a rat study, Haddad & Adams (2002) measured myogenin mRNA after one exercise bout or after 2 exercise bouts 48 hours apart. After one exercise bout, myogenin was increased from 40 hours post-exercise. The myogenin response 12 hours after 2 bouts was of the same magnitude as 40 hours after one bout, indicating that this portrayed the response to the first exercise bout. Summation of the responses of the two bouts only occurred at 40 hours after the 2nd bout (Haddad & Adams, 2002). In summary, it appears myogenin mRNA is not upregulated in rats for several hours after exercise. However, human studies have shown increased expression of myogenin mRNA 30min (Willoughby & Nelson, 2002), 6 hours (Psilander *et al.*, 2003), 8 hours (Yang *et al.*, 2005), and 12 hours (Bickel *et al.*, 2005) after resistance training. However, one study also indicated no increase in the days after resistance training (Crameri *et al.*, 2004). Since I measured myogenin at the protein level, I will focus my discussion on the studies that also report changes in muscle myogenin content. Myogenin protein content has been observed to increase 6 hours after resistance training in one study (Willoughby & Nelson, 2002) but not at all within 24 hours in another study (Bamman *et al.*, 2004). Considering all the above studies, I can conclude that in my study, myogenin protein increases would not be influenced by the second

exercise session, but are reflecting responses to the first training session 48 hours earlier. These responses should thus be viewed as responses ~50 hours (or roughly 2 days) after a single bout of HIT.

Myogenin protein expression at this time varied considerably between subjects and neither of the groups showed a consistent increase in myogenin protein expression at this stage. The most important observation after acute HIT was the strong correlation between the myogenin response in DH subjects and the extent of perceived pain (r = 0.98). Subjects that experienced the most severe leg pain consequently had the highest myogenin protein expression. Previous animal (Darr & Schultz, 1987;Tiidus *et al.*, 2005) and human studies (Crameri *et al.*, 2004) have shown satellite cell proliferation after acute eccentric exercise, and thus the changes in myogenin associated with pain most likely reflect satellite cell differentiation. The fact that Bamman *et al* (2004) found no changes in myogenin protein after resistance training may reflect a lack of damage in response to the exercise protocol or that the biopsy time point (24 hrs post exercise vs. 48 hrs in my study) was too early to reveal changes.

Changes in myogenin protein expression after 4 weeks HIT

After 4 weeks of HIT, myogenin protein expression increased by 84% in UP and 63% in DH groups. The absence of a group effect lead us to conclude that 4 weeks of high intensity training produces an increase in myogenin protein expression, irrespective of the mode of muscle contraction. The more complex issue at hand is the origin and function of the increase in myogenin protein, and whether this could differ with mode of muscle contraction.

Currently there is little literature concerning myogenin expression after long periods of training. After 10 weeks of resistance training, Hespel *et al.* (2001) reported a 60% increase in myogenin muscle protein that coincided with a modest increase in *quadriceps*

CSA. Interestingly, greater hypertrophy in a creatine supplementation group did not correspond with any increased myogenin expression, questioning the necessity of myogenin for muscle hypertrophy (Hespel *et al.*, 2001). In a similar study, Willoughby and Rosene (2003) observed increases of 35% and 15% in muscle myogenin protein after 12 weeks of resistance training with or without creatine supplementation, respectively. As no markers of myogenesis apart from MRFs were measured in these studies, I cannot be certain regarding the function and origin of these factors. In my study, the fact that 4-week myogenin results were similar in UP and DH groups, but satellite cell proliferation differed, suggests a myonuclear origin, or at least in part.

Results from various animal studies reveal more concerning the origin and function of myogenin during muscle adaptation. Adams *et al.* (1999) used antagonistic muscle ablation to induce compensatory muscle hypertrophy. Myogenin and p21 (inhibitor of cell cycle progression and initiator of differentiation) mRNA were upregulated from 6 hours and returned to baseline at 12 days, correlating closely, thus indicating that myogenin is derived from satellite cells and not post mitotic myonuclei. The authors argued that rapid expression of myogenin could be attributed to the immediate differentiation of some satellite cells (Adams *et al.*, 1999). Carson and Booth (1998), using a stretch-overload hypertrophy model, found an elevated and sustained increase in myogenin from 3 days to 21 days of increased loading. They concluded that myogenin was necessary for the hyperplasia seen in the avian model they used (Carson & Booth, 1998). Lowe & Alway (1999) also investigated stretch-overload induced hypertrophy in an avian model. Muscle hypertrophied despite the irradiation of satellite cells, and levels of MRF4 and MyoD increased to the same magnitude as with satellite cells. Only myogenin mRNA was significantly lowered (although still elevated compared to baseline) after 3 days in satellite cell deficient muscle, confirming that satellite cells contribute to the increase in myogenin mRNA, but also suggesting a contribution by myonuclei.

Bickel *et al.* (2005) examined the myogenic response to one and two acute bouts of resistance training. Myogenin mRNA increased from 12 to 48 hours after the first exercise bout and upregulation was continued for 24 hours after the second bout. 48 hours after the second bout myogenin decreased back to baseline values. A correlation between myogenin and p21 were seen as evidence that elevated myogenin mRNA reflected differentiating satellite cells (Bickel *et al.*, 2005). The results from this study implies that satellite cell differentiation can be switched on very quickly and switched of again as quickly in response to training. This underlines the importance of regular and progressive training to elicit a chronic myogenin response. Since myogenin is a MRF, it is also if interest what other proteins are affected downstream.

In one of the most comprehensive rat studies concerning myogenesis to date, Putman *et al.* (2000) subjected rats to daily chronic low frequency stimulation for 5, 10 and 20 days without evidence of muscle damage. Satellite cells, PCNA (proliferating cell nuclear antigen, a marker for proliferation) and myogenin staining were elevated similarly (4x) after 5 and 10 days. This was followed by an increase in embryonic MyHC from 10 to 20 days. Satellite cells staining positive for myogenin were more prevalent in cells stained for type IIB and embryonic MyHC than in cells stained for type I and IIa MyHC. The authors concluded that the upregulation of satellite cells and myogenin precedes the conversion of MyHC phenotype, and speculated that a higher myonuclear density may be required for the change to a slower phenotype (Putman *et al.*, 2000). This study thus outlined the function of myogenin in both myogenesis and fibre type conversion, and could explain elevations in myogenin protein observed in previous studies.

Exercise is not the only stimulus for MRFs. When rats were supplemented with clenbuterol (anabolic steroid) for 4 weeks, myogenin mRNA decreased while MyoD increased together with an increase in MyHC type IIx (Mozdziak *et al.*, 1998). In rats subjected to treadmill running 4 times a week for 8 weeks, myogenin mRNA and protein

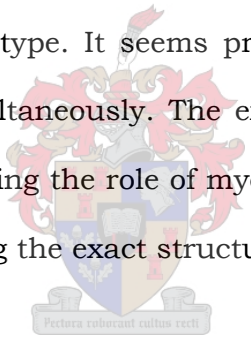
increased and correlated with citrate synthase activity while myoD stayed at basal levels (Siu *et al.*, 2004). These results suggest that levels of MyoD and myogenin are associated with MyHC composition and cell metabolism. The relationships between myogenin and a slower MyHC phenotype (type I & IIa) and between MyoD and a faster phenotype (type IIx) have been shown in both animal (Hughes *et al.*, 1993) and human muscle samples (Vissing *et al.*, 2005;Willoughby & Nelson, 2002). Although MyHC conversion to a slower phenotype was not evident in my study, the myogenin elevations suggest that this may have taken place if the study duration had been more prolonged.

Recently two human studies investigated the early changes in myogenic regulatory factors in response to endurance exercise. Yang *et al.* (2005) reported no changes in myogenin mRNA during the first 24 hours after a 30 min run at 75% $V0_2$max. In contrast Kadi *et al.* (2004a) found an immediate increase in myogenin staining in myonuclei after an endurance cycling session. The extent of the increase was such (1- 3% of myonuclei expressing myogenin) that it would probably not be picked up by muscle homogenate protein analysis. The authors proposed that myogenin protein expression in myonuclei represented the activation of pathways controlling aerobic muscle metabolism (Kadi *et al.*, 2004a), and confirmed that it is not expressed solely by differentiating satellite cells.

Very few studies have investigated myogenesis specifically in response to eccentric exercise. One human study by Crameri *et al.* (2004) subjected subjects to a high intensity eccentric resistance training bout and investigated responses over the following 8 days. Although satellite cells proliferated, no changes in myogenin or embryonic MyHC were observed. The authors concluded that one bout was insufficient to induce satellite cell differentiation (Crameri *et al.*, 2004). My study indicates that this is not the case if the eccentric stimulus is DH running and if sufficient DOMS is experienced. Peters *et al.* (2003) studied responses to an acute bout of stimulated eccentric exercise over the subsequent 10 days, but using a rat model. Myogenin mRNA increased within 3 hours

and peaked at 24 hours post exercise. Large as well as very small, possibly new or regenerating fibres were stained positively for embryonic MyHC from 2 days after exercise to 7 days afterwards. The authors concluded that satellite cells were activated, differentiated, and incorporated into existing fibres in this time (Peters *et al.*, 2003). This conclusion assumes that appearance of embryonic MyHC in adult muscle implies satellite cell migration.

From these results, it appears that myogenin plays a multi-functional role in muscle's response to training. Firstly, myogenin influences hypertrophy through the differentiation of satellite cells into myoblasts and secondly, it stimulates hypertrophy through other independent mechanisms in adult myonuclei. Additionally, myogenin may influence MyHC expression through expression in myonuclei, which may lead to a conversion to a slower fibre phenotype. It seems probable that myogenin can perform more than one of these roles simultaneously. The extent and time course of myogenin expression may be helpful in revealing the role of myogenin in a given circumstance, but this can only be done by pinpointing the exact structures and areas of expression.



In the present study increases in myogenin were noted despite a lack of muscle hypertrophy. In addition, myogenin did not correlate with changes in MyHC isoform proportions. The HIT intervention in this study cannot be compared directly to resistance training, as it combines elements of overload (increased force production) with that of anaerobic running. In a similar fashion, HIT can not be compared to endurance training in untrained athletes, although there are some mutual responses. The increase in myogenin in the downhill group is most probably a result of satellite cell proliferation and differentiation, as I have shown increased satellite cell frequency following HIT. The reason for satellite cell proliferation was discussed previously (section 6.3.3).

The function of myogenin upregulation in UP subjects may be even more complex to explain. It is widely accepted that eccentric but not concentric contractions cause exercise-induced muscle damage (Gibala *et al.*, 2000;Newham *et al.*, 1983). In the present study uphill running did not induce a significant increase in satellite cell frequency, although a non-significant trend to increase by 4 weeks was observed. It is thus likely that myogenin protein expression, at least in part, is not the result of increased satellite cell differentiation. Since no changes in MyHC isoforms or fibre area were seen, it is difficult to explain this event. Possibly myogenin gene expression was switched on as a signal for hypertrophy or fibre type transformation, but 4 weeks of uphill training was not sufficient to provoke a measurable change. As mentioned earlier, future research into the myogenic response to exercise will need to concentrate on immunohistochemical analysis of MRFs as this clearly shows the origin of increased MRF protein. Also, training interventions would need to have a longer duration.

6.4 MORPHOMETRY

Muscle fibre cross-sectional area

The absence of change in mean fibre CSA after 4 weeks of HIT was not unexpected. Firstly, responses in already trained athletes tend to be smaller in magnitude than those in untrained subjects (Kubukeli *et al.*, 2002). Secondly, the training intervention may have been too short to provoke changes in fibre area, and finally the HIT stimulus may not have been sufficient to provide further increases in muscle size in already trained endurance athletes. Nevertheless, after 4 weeks of HIT, a significant reduction in the standard deviation from the mean was observed for both UP and DH groups. This may indicate that although mean CSA did not change, there was a shift in fibre diameter towards the mean. This shift suggests that small fibres, possibly type I fibres hypertrophied while big fibres, possibly type IIx fibres, decreased in size. This possible

difference may suggest conflicting signals, with different outcomes, may have been operating simultaneously. As I did not quantify fibre type in histological sections, I can only speculate regarding such an adaptation. Such an adaptation could be warranted as novel eccentric exercise recruits mainly fast fibres (Moritani *et al.*, 1987), and chronic eccentric exercise is thought to result in increased recruitment of slow fibres (Nosaka & Clarkson, 1995). This could imply that chronic eccentric exercise stresses type I fibres and consequently induced them to hypertrophy. However, since the standard deviations decreased in UP as well, it is likely the speed of training or HIT per se that induced a change in fibre size, instead of the type of contraction.

MyHC isoform proportions

No changes were seen in MyHC isoform proportions after the training intervention in either group. Similar to the fibre area, a change in MyHC proportions was not anticipated after only 4 weeks of HIT training in already trained endurance athletes. After 6 weeks of high intensity uphill training, moderately trained endurance athletes showed no signs of fibre type conversion (Houston & Thomson, 1977). Even 12 weeks of resistance training could not induce a change in fibre type proportions in endurance trained cyclists (Bishop *et al.*, 1999). Only one previous study has shown a modulation of MyHC proportions after high intensity training in trained athletes. This study showed a decrease in type IIb fibres and an increase in type IIa and IC fibres (Kraemer *et al.*, 1995). However, subjects were only military trained (not true endurance athletes) and the training intervention lasted 12 weeks.

The fibre type modulation towards a predominant type IIa muscle in response to concentric resistance training is well founded (Campos *et al.*, 2002;Jurimae *et al.*, 1996). However, changes in response to eccentric resistance exercise are more controversial (Friedmann *et al.*, 2004;Paddon-Jones *et al.*, 2001;Raue *et al.*, 2005). Moreover, fibre

type changes to HIT of either an eccentric or concentric nature is largely unknown. To investigate this, longer training interventions should provide better results.

6.5 CHANGES IN RUNNING PERFORMANCE PARAMETERS AFTER 4 WEEKS HIT

This was the first study to assess changes in performance parameters after repetitive downhill running at high intensities. However, the discussion that follows must be considered on the background of the fact that training volume decreased at the same time that intensity increased. VO_2max values did not change for either the inclined or the level incremental exercise test to exhaustion. The absence of an improvement in VO_2max after downhill HIT can be explained by the fact that downhill running requires considerably less oxygen consumption than level running (Liefeldt *et al.*, 1992;Minetti *et al.*, 2002) and therefore DH HIT did not strain the aerobic energy system. The lack of improvement of VO_2max in UP subjects was less expected, though not surprising. Previously, 6 weeks of intermittent uphill training in moderately trained runners also did not lead to improvement in VO_2max (Houston & Thomson, 1977). Similarly, high intensity interval training at the velocity of VO_2max has failed to induce changes in VO_2max in well-trained runners (Acevedo & Goldfarb, 1989;Billat *et al.*, 1999;Smith *et al.*, 2003). Uphill running includes a greater component of anaerobic training than level running (Olesen, 1992). This is apparent in the present study when one considers the inability of subjects to complete the 3 min intervals and the % HR maximum of above 95%. Despite no improvement in maximum oxygen consumption, all these studies mentioned above reported improvements in simulated race performance after 4 - 8 weeks of HIT.

In my study I used PTS and 10 km running time as indicators of performance. Although PTS correlates with VO_2max, it may be more adaptable (Noakes *et al.*, 1990) because it

increases linearly in incremental tests, whereas VO_2max may plateau. I hypothesized that PTS during the inclined exercise test to exhaustion would improve in UP subjects after training, since training was of similar inclination as the inclined exercise test to exhaustion and undertaken at high speeds. However, an increase in $PTS_{incline}$ was observed for both groups combined. The improvement did not reach significance in the separate groups, but this may be due to small subject numbers. Nevertheless, this result implies that HIT improves uphill running ability, whether or not the HIT was done as UP or DH training. I cannot exclude a possibility that subjects improved their peak treadmill test due to 'psychological factors' such as the test being the last of a series of tests (Laursen & Jenkins, 2002). However this is unlikely, as our laboratory have previously shown $PTS_{incline}$ to be a repeatable test-parameter (unpublished data, abstract in Appendix *iv*). This is a finding of some significance, as it is the first to suggest that the downhill component of high intensity endurance running is possibly an important component of the adaptive process for increased performance.

Previously, eccentric cycling for 6 - 8 weeks has resulted in substantial improvements in eccentric strength-endurance assessed by maximal work performed in a time period (Friden *et al.*, 1983a) as well as increased isometric leg strength (1 RM) (LaStayo *et al.*, 1999;LaStayo *et al.*, 2000). Although the muscle adaptations in response to eccentric training resulting in such improvements in performance are unclear, they may include changes in muscle fatigue resistance (Michaut *et al.*, 2004;Willems & Stauber, 2002), improved angle of torque production (Brockett *et al.*, 2001), or even superior sarcomere organization (Friden, 1984;Yu *et al.*, 2004). The molecular mechanics of eccentric contraction are extremely complex and fall beyond the scope of this study (briefly mentioned in section 2.1.2). However, I suggest that sarcomere level adaptations including re-organisation with accompanying satellite cell activation and proliferation are likely.

PTS has been shown to be a strong predictor of running performance (Noakes *et al.*, 1990). In addition, in a pilot study I have previously shown that PTS during an inclined incremental test to exhaustion is a good predictor of running performance in sub-elite athletes (conference proceedings, abstract in appendix). Accordingly, one might expect that changes in inclined PTS should coincide with changes in 10 km running time. However, running time was unaltered in UP subjects and decreased significantly in DH subjects. Several factors may be responsible for this reduction in field test performance. Firstly, the post-HIT 10 km time trial performances were not a true reflection of the running performance of the athletes, because uncontrollable weather circumstances (a strong head-wind in one race and a heat wave in another) affected running conditions during both of the two post-intervention staged time trials. However, it is also possible that the decreased time trial performance was a real effect attributable to the significant reduction in training volume during the month of HIT. This would explain why only DH subjects decreased time trial performance. Although UP subjects also decreased their training volume, their HIT was significantly more intense, while DH training did not put equivalent strain on the cardiovascular system. Finally, decreased time trial performance could have been a product of insufficient recovery time after the strenuous 4-week HIT programmeme. Usually a 6-8 day taper is sufficient to improve performance (Mujika *et al.*, 2000) or at least maintain performance relative to before-taper conditions (Shepley *et al.*, 1992). However, in the present study, subjects in both UP and DH groups were noticeably fatigued by the end of the training intervention, as subjects were unaccustomed to HIT, and especially the frequency of HIT (3 times / week). Thus, I speculate that with more time to recover subjects may have improved performance, despite environmental conditions.

6.6 CONCLUSIONS

In summary, the present results show that skeletal muscle was influenced by 4 weeks of high intensity downhill running, responding with an increase in satellite cell frequency. Changes in satellite cell frequency were not clearly evident after 4 weeks of high intensity uphill running using a similar training intervention protocol. More consistent responses may have been obtained using individuals who were not previously trained, but the objective was to determine specifically if already trained subjects would respond to a change in training. The downstream results of the increased satellite cell population are not understood completely, and further research is necessary to clarify this. However, I could clearly show that muscle myogenin protein increased during both uphill and downhill HIT. Myogenin protein most likely reflected changes in satellite cell proliferation and differentiation in DH subjects, while possibly reflecting an early signal for either hypertrophy or fibre type transformation in UP and DH subjects. However, a more prolonged protocol would be required to determine if those would indeed be downstream responses since myosin heavy chain isoform proportions and mean fibre cross-sectional area remained unaltered despite HIT training. Peak treadmill speed during an inclined incremental test to exhaustion increased in both training groups, implying that DH training may hold similar advantages to UP training regarding running performance. This phenomenon was not confirmed by 10 km running times. It was concluded that changes in performance parameters may become more pronounced and changes in morphology may become visible in response to a longer training intervention. The most important finding that I report for the first time is that muscle responds to 4 weeks of eccentrically biased training with an early and sustained elevation in satellite cell number.

CHAPTER 7: LIMITATIONS AND FURTHER RESEARCH

I successfully recruited 13 moderately to well-trained distance runners who were divided into two training groups which were successfully matched for training volume and 10 km running time. Due to the difficulty of recruiting subjects to undergo multiple biopsies I could not recruit any more subjects. Ideally, the study design would have been much improved if there was a control group to continue their normal training between the pre and post 4-week training phase. However, by having an UP HIT group, the effect of training intensity could be separated from the effect of increased exposure to DH running.

Despite my attempts to recruit subjects with similar training volume, a reasonable amount of intra-subject variation existed within the training groups with respect to running experience. While some subjects had been training for several years and included regular HIT into their training (fartlek, tempo runs, track sessions), a few of the subjects were novice runners, who had been training systematically for less than a year and never included any high intensity training in their routine. I was not oblivious to this when recruiting subjects, but set out specific criteria regarding 10 km race time and training volume to limit differences between athletes. It would have been a nearly impossible task to recruit more uniform subjects in the small community in which I did the study. I conclude that 2 HIT training sessions per week are within the capacity of athletes already well-trained to tolerate distance running. But I provide added evidence that 3 HIT sessions per week require more baseline training, some previous HIT experience, or the use of recovery modalities between sessions.

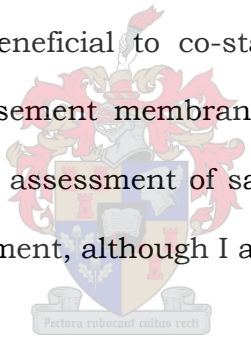
In retrospect, the intensity of UP HIT was too high, and several UP subjects could not complete their prescribed training per session. However, it was difficult to design a

correct training protocol for UP training since training had to be similar in duration to DH training, but of sufficient speed to induce high-intensity adaptive responses rather than slow, resistance-induced responses. Also, there is very little literature on either UP or DH HIT. Although I experimented with the training intensity before the start of the study, I could not anticipate the difference in response to training between subjects. Usually, uphill training comprises of much shorter running bouts than in the present study, typically including 100-200 m intervals with several repetitions and sets. It may have been advantageous to have done more preliminary testing of the protocol for the uphill training programme in more subjects, to ensure that UP subjects had the same metabolic intensity (% HRmax) as DH. However, I succeeded in producing a sufficient HIT stimulus in both groups, with a training HR of at least above 90% and with similar RPE ratings, indicating that I succeeded to some extent in creating training loads that elicited a high intensity effort.

All subjects in the study were students at the local university and all testing had to be done within a semester to exclude possible detraining effects over holidays. This placed strict time limitations on the study design. For instance, more time after the training intervention would have allowed a) subjects to rest sufficiently before post-training testing, b) laboratory performance tests to exhaustion to be done in duplicate to ensure their repeatability, and c) field tests to be repeated on days with more favourable environmental conditions.

Another possible limitation of the study design was that baseline muscle biopsies were not taken 4-6 hours after a training session, similar to the subsequent two biopsies. As argued in the discussion, due to the large variation in biopsy-timing after the most recent exercise bout (especially at baseline, but also some variation post-HIT), it was not possible to measure any variables with a short half-life that are known to be upregulated within minutes (or even one or two hours) after exercise. This limited my options to

investigate other proteins after acute exercise, such as IGF-1 splice variants and inflammatory markers such as $NF_{kappa}B$. Despite this I may still analyse less labile markers of muscle damage, inflammation and MRFs in the biopsy samples from this study. Such research may lead to a better understanding of the mechanisms behind, and the consequences of increased satellite cell proliferation in the two groups. It is possible that 'responders' and 'non-responders' exist regarding the extent of satellite cell proliferation in response to exercise, and that this contributed to the large subject variability. An increased sample number can possibly point out such a trend. Further research into the role of MRFs, and especially myogenin, should focus on the immunohistochemical quantification of protein expression, as this will reveal more about their function than only measuring muscle protein content. In addition, measuring changes in the proportion of hybrid fibres may provide further insight into possible fibre type changes. It would also be beneficial to co-stain with a sarcolemma associated protein (e.g. dystrophin) and a basement membrane associated protein (e.g. laminin). This would enable more qualitative assessment of satellite cell cytoplasmic domain and unquestionable quantitative assessment, although I am confident of my quantification.



The present study asked more questions than it answered, and therefore leaves much room for further research. Downhill running, and especially repetitive downhill running, is generally regarded as a risk factor for injuries. This study has shown the positive side of downhill running, however, more research into the advantage of downhill running is necessary before it can be practically applied. With an improved study design and by incorporating more comprehensive laboratory analysis, this model can teach us a great deal about the regulation of the human satellite cell population. If research can elucidate the different triggers for satellite cell proliferation and incorporation into muscle, it may lead to various practical applications. If the body's satellite cell pool can be utilized at will, it may lead to improved training adaptations and reductions in disease-associated atrophy.

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***i)* Validation of training volume**

To ensure subjects did not report exaggerated training volumes when measured by a questionnaire and an interview, 8 subjects were given HR monitors and training logbooks to monitor their training for 7 days of their normal training. Assessing training by these 2 different methods correlated significantly (p = 0.001, Fig. 7.1) and thus the use of training volume assessed by questionnaire seem to be warranted. No statistical difference was found between the two methods when compared by a dependant samples t-test.

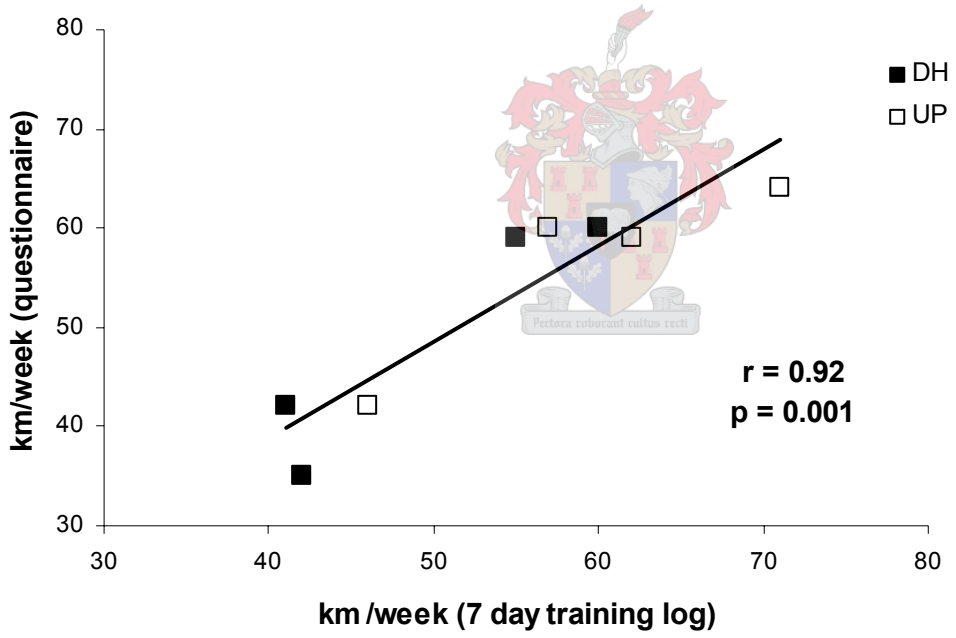


Fig. 1 ***Training volume measured by questionnaire versus training volume measured by 7-day training log***

ii) Biochemistry

Table 1 ***Lysis buffer***

Reagent	Concentration
Tris/HCl, pH 7.4	2.5 mM
EDTA, pH 7.4	1 mM
EGTA, pH 7.4	1 mM
mannitol	250 mM
NaF	50 mM
NaPPi	50 nM
PMSF (in methanol)	0.1 mM
SBTI	4 µg/ml
leupeptin	1 µg/ml
bensamidine	1 mM
DTT	1 mM
NP40	1%
SDS	0.1%
Na deoxycholate	0.5%

Table 2 ***Phosphate buffered saline***

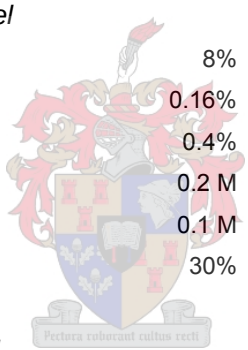
Reagent	Concentration
NaCl	0.8% w/v
KCL	0.02% w/v
Na_2HPO_4	0.144% w/v
KH_2PO_4	0.024% w/v
dH_2O	

Table 3 *Sample buffer* (Laemmli, 1970)

Reagent	Concentration
Tris-HCL	62.5 mM
glycerol	25%
SDS	2%
ß-mercaptoethanol	5%
bromophenol blue	0.01 %

Table 4 *Separating and stacking gel*

Reagent	Concentration
Separating gel	
acrylamide	8%
bis-acrylamide	0.16%
SDS	0.4%
Tris (pH 8.8)	0.2 M
glycine	0.1 M
glycerol	30%
Stacking gel	
acrylamide	4%
bis-acrylamide	0.08%
SDS	0.46%
Tris (pH 6.8)	0.125 M



iii) Validation of co-staining

Co-staining for CD56 and M-cadherin was done using a 3-step method (primary antibody – secondary biotinylated antibody – tertiary fluorescent marker) for both antibodies. It is possible that this method can create false positive staining for M-cadherin (which was always the second protein assessed) if the secondary antibody bound to CD56 remains unsaturated after the binding of the fluorescent marker. This is unlikely, as fluorescent marker concentrations are supposed to saturate the secondary antibody. However, to ensure this was the case, and all M-cadherin quantification was valid, I stained some slides with a 2-step CD56 (primary antibody – fluorescent secondary antibody) and a 3-step M-cadherin protocol. Validation included immunostaining of 9 biopsy samples, one from baseline, one after two sessions and one after 4 weeks for 3 subjects. Results show no difference between these two staining protocols at any of these time points. Fig 2 illustrates these findings.

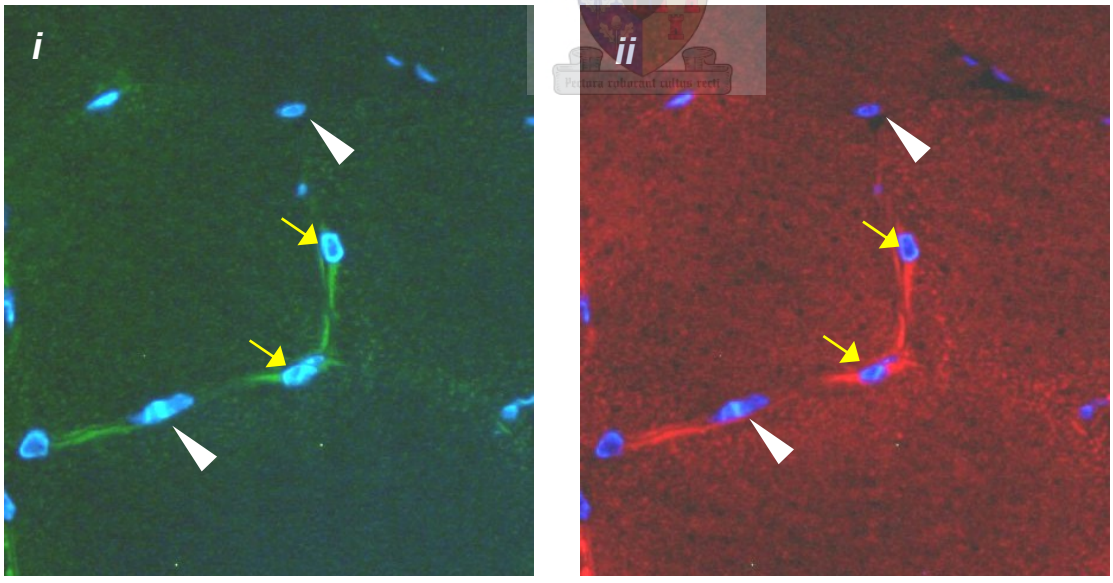


Fig. 2 ***Validation of co-staining for CD56 and M-cadherin during immunohisto-chemical identification of satellite cells*** *Panels i & ii* show the same magnified portion of a muscle section stained for *i)* CD56 using a 2-step protocol and, *ii)* M-cadherin using a 3-step protocol (40x). Yellow arrows identify satellite cells, which can be distinguished from myonuclei within the sarcoplasm (white arrow heads) by the surrounding fluorescent staining and its position outside the sarcolemma.

iv) Evidence of myogenesis

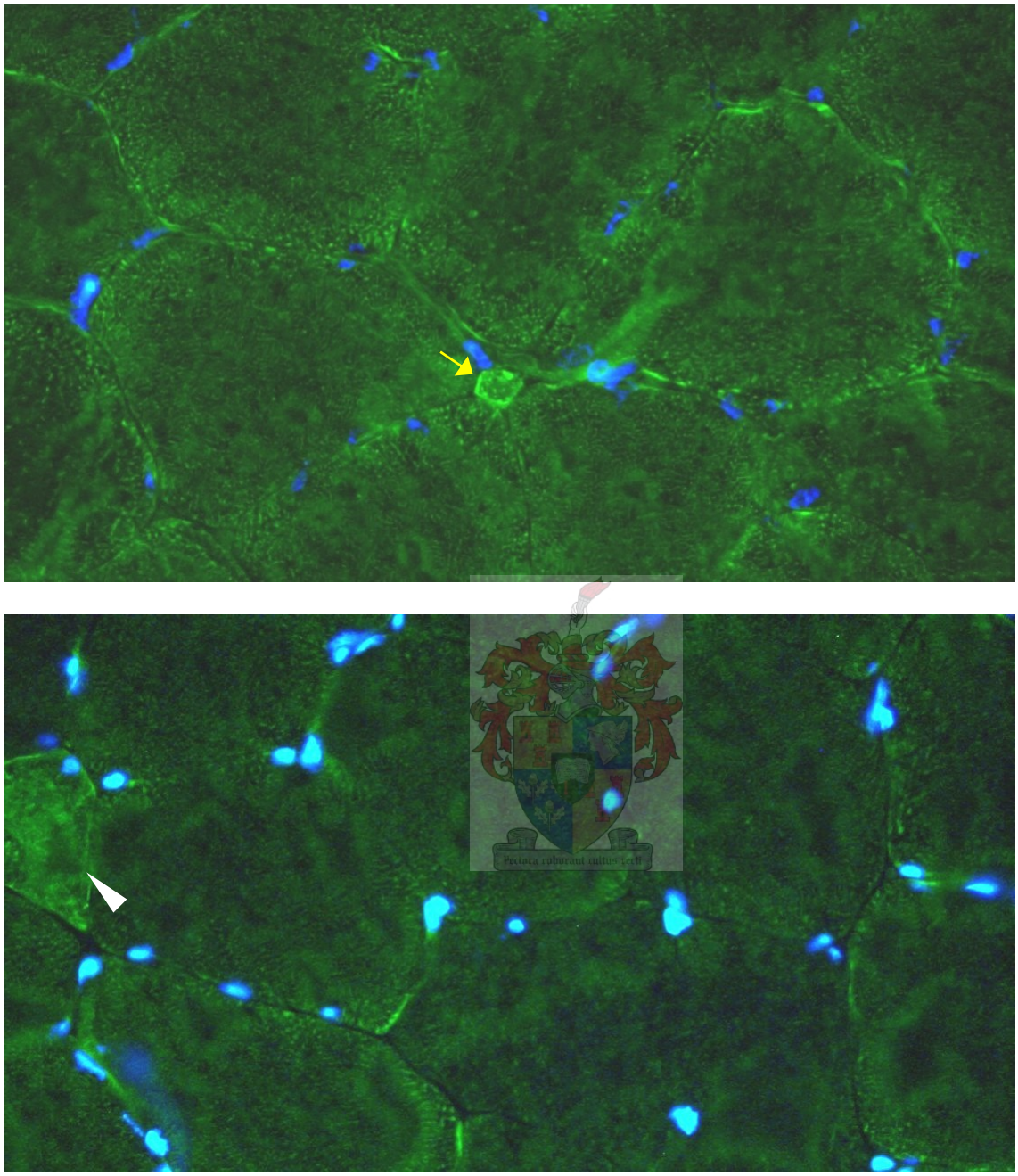


Fig. 3 ***Possible evidence of myotubes and small fibres staining positively for CD56*** *Panels i & ii show* magnified portions of a muscle section stained for CD56. The yellow arrow points to a structure identified as a possible myotube. The white arroww heads shows a small muscle fibre staining positively for CD56.

***v)* Unpublished data referred to**

In the discussion I quoted my own pilot study which has not been published apart from in the proceedings from the Physiological Society of Southern Africa's annual conference (2003). Therefore I provide the abstract:

FLAT AND GRADIENT LABORATORY TREADMILL TESTING AS PREDICTORS OF 10 KM PERFORMANCE

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Peak treadmill speed (PTS) is the best predictor of marathon performance. However, 10km races may require more speed and power. Racing data are often obtained using questionnaires, resulting in race performances reported over different courses for different subjects and frequently not concurrent with laboratory testing. Therefore, our aim was to determine if treadmill testing on a gradient would be a good predictor of 10km road race performance (RR) on a specifically constructed course that was raced in the same time period as laboratory testing. We recruited 15 competitive, male distance runners who performed horizontal and gradient (8.5%; 5°) $V0_2max$ testing (66.4 ±4.4 and 67.0 ± 4.7 mL/kg/min, respectively). PTS was 20.9 ±1.1 km/h and 15.7 ± 0.8 km/h, respectively. Training volume reported by questionnaire was 57.8 ±22.7 km/week. $V0_2max_{flat}$ correlated with athletes' personal best (PB) 10 km time over the previous 6 months ($r= -0.58$, $P<0.05$), but correlated better with RR performance ($r= -0.66$, $P<0.01$). Similarly, PTS_{flat} correlated better with RR than PB? ($r= -0.93$, -0.81 $P< 0.001$) and $V0_2max_{grad}$ correlated better with RR than PB ($r= -0.74$, $P<0.001$; $r= -0.57$, $P<0.05$), and $PTS_{gradient}$ correlated better with RR than PB ($r= -0.73$, $P<0.005$; $r= -0.59$, $P<0.05$). Training volume correlated poorly with RR ($r= -0.53$, $P<0.05$), and not with PB. Our data show conclusively that a) PTS_{flat} and $V0_2max_{grad}$ were more closely associated with performance than $PTS_{gradient}$ and $V0_2max_{flat}$ and b) race times over the same course and during the testing period are more closely associated with laboratory results than personal best times for the preceding 6 months or reported training volume. However, quantification of training by questionnaires may be inaccurate.