The comparison between two high-intensity interval training protocols on skeletal muscle and satellite cell dynamics

by

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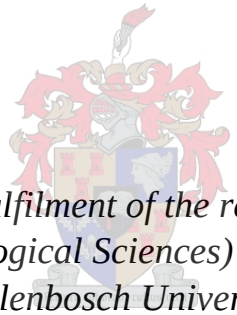

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Declaration of originality

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Opsomming

Inleiding: Hoë intensiteit-interval oefening (HIIO) intervensies is populêr by lang afstand atlete vir spierkrag, top spoed en arobiese kapasiteit. Hardloop betrek die gebruik van beide esentrise en konsentriese kontraksies, met die vlak van die oppervlakte beoordeel die verhouding tussen die twee. Om afdraand te hardloop word beskou as esentrise gebeseerde oefening. Aan die ander kant om opdraand te hardloop word beoordeel as konsentries gebeseerde oefening. Om opdraand en afdraand te hardloop is dus twee verskillende rolspelers in spier aanpassing, alhoewel beide kan potentieel deur beide gereguleer word deur satelietsel dinamieka.

Hipotese: Die verskillende weergawes van HIIO se resultaat sal verskillende skeletale spier skade wees.

Metodes: Twaalf gesonde aktiewe mans was ewekansig in beide afdraand haardloop (AHG) of 'n opdraand hardloop gegropeer (OHG). Die deelnemers het deur die basislyn en voltooide prestasietoetse wat bestaan uit 'n plat VO_{2max} trapmeul toets, maksimaale isometriese sterkte toets en 5km pad tyd toets (TT). Die oefeninge het bestaan uit 10 HIIO sessies oor 'n tydperk van 4 weke. Elke sessie het bestaan uit 6 intervalle van +5% gradient en 80% van piek trapbandspoed of -10% gradient en 90% piek trapbandspoed. Spierbiopsie en bloed monsters was geneem tydens basislyn, asook 6 ure na die eerste en die laaste sessie.

Resultate: Prestasietoetse: OHG maar nie die AHG, het verbeter VO_{2max} van die basislyn (59.48 ± 1.73 ml.kg.min^{-1} – 61.86 ± 1.28 ml.kg.min^{-1}). Die AHG maar nie die OHG, het die maksimale isometriese verbeter na die 4 weke van HIIO (734 ± 133 N - 893 ± 55 N). Beide groepe het hulle 5km TT prestasie verbeter met $3.5 \pm 1\%$. Die AHG maar nie die OHG het 'n merwaardige verbetering in CK vlakke 6 ure na hardloop ($p < 0.05$). Maskulere reaksie na 4 weke van HIIO vir die AHG het 'n verbetering ingesluit dursnit ($p < 0.05$), verbeterde satelliet sel groep grootte (0.1 ± 0.001 satelliet sel/vesel - 0.3 ± 0.02 satelliet sel/vesel), en 'n verbetering in mioD na die eerste oefening ($p < 0.05$). Muskulere aanpasings in die OHG het 'n vertbeterde kapilêre tot vesel verhouding ingesluit (1.76 ± 0.18 kapilêre/vesel – 2.55 ± 0.20 kapilêre/vesel) en kapilêre digtheid (249 ± 39 mm^2 – 304 ± 57 mm^2) met oefening.

Afsluiting: Vier weke van opdraand of afdraand HIIO het gelei tot fisiologiese aanpassing deur middle van verskillende meganismes, een met verhoogte aktiwiteitheid van sateliet selle en 'n skerker spiersamertrekking en die ander wat spierperfusie en suurstof gebruik behels. Die aanpassingsmeganismes is opleidingspesifiek, maar hulle lei albei in 'n soortgelyke verbetering in 5km-renprestasie.

Abstract

Introduction: High intensity interval training (HIIT) interventions are popularly used by endurance athletes to increase muscle strength, peak speed and aerobic capacity. Running involves the use of both eccentric and concentric contractions, with the level of the running surface determining the ratio between the two. Downhill running is considered eccentric-biased exercise. Conversely uphill running is considered concentric-biased exercise. Uphill running and downhill running are therefore two different role players in muscle adaptation, although potentially both act through regulating satellite cell (SC) dynamics.

Hypothesis: The different modes of HIIT will result in differing skeletal muscle damage, satellite cell activity and morphological adaptation, resulting in differing muscle adaptation, aerobic capacity, muscle strength and running performance.

Methods: 12 healthy active males were randomized into either a downhill running (DHG) or an uphill running group (UHG). Subjects underwent baseline and post training performance testing which consisted of a flat VO_2max treadmill test, maximal isometric strength test and a 5km road time trial. Training consisted of 10 HIIT sessions over a period of 4 weeks. Each session consisted of 6 intervals at either +5% gradient and 80% peak treadmill speed or -10% gradient and 90% peak treadmill speed. Muscle biopsies and blood draws were taken at baseline, as well as 6 hours after the first and the last session.

Results: Performance testing: The UHG, but not the DHG, improved VO_2max from baseline (59.48 ± 1.73 ml.kg.min^{-1} – 61.86 ± 1.28 ml.kg.min^{-1}). The DHG, but not the UHG, improved maximal isometric after the 4 weeks of HIIT (734 ± 133 N - 893 ± 55 N). Both groups improved their 5km TT performance by 3.5 ± 1%. The DHG but not the UHG had a significant increase in CK levels 6 hours after running ($p < 0.05$). Muscular response to 4 weeks HIIT for the DHG included an increased CSA ($p < 0.05$), increased SC pool size (0.1 ± 0.001 SC/fibre - 0.3 ± 0.02 SC/fibre), and an increase in myoD after the first bout of exercise ($p < 0.05$). Muscular adaptations in the UHG included increased capillary to fibre ratio (1.76 ± 0.18 – 2.55 ± 0.20) and capillary density (249 ± 39 mm^2 – 304 ± 57 mm^2) with training.

Conclusion: Four weeks of uphill or downhill HIIT resulted in physiological adaptation by different mechanisms, one by enhanced SC activity and a more forceful contraction and the other involving muscle perfusion and oxygen utilization. The mechanisms of adaptation are training specific, yet they both result in a similar improvement in 5km race performance

Table of contents

List of abbreviations

ADP	Adenosine diphosphate
ANG	Angiopoietin
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BDNF	Brain derived neurotrophic factor
BSA	Bovine serum albumin
C:F	Capillary to fibre ration
CD	Capillary density
CFPE	Capillary-to-fibre perimeter exchange index
CK	Creatine kinase
CSA	Cross sectional area
DOMS	Delayed onset of muscle soreness
ECM	Extracellular matrix
FGF	Fibroblastic growth factor
HGF	Hepatocyte growth factor
HIIT	High intensity interval training
HR	Heart Rate
HSP72	Heat shock protein 72
IF	Intermediate filaments
IGFBP	Insulin-like growth factor binding protein
IGF-I	Insulin-like growth factor
IL-6	Interleukin-6
JAK	Janus-activated kinase
LDH	Lactate dehydrogenase
LIF	Leukaemia inhibitory factor
L_T	Lactate threshold
MAPK	Mitogen-activated protein kinase
MGF	Mechano-growth factor
MHC	Myosin heavy chain
MMP	Matrix metalloproteinase
MND	Myonuclear domain

MRF	Myogenic regulatory factors
mTOR	Mammalian target of rapamycin
MURF-I	Muscle RING-finger protein-I
NSAIDS	Non-steroidal anti-inflammatory drugs
Pax7	Paired box protein 7
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
RIPA	Radioimmunoprecipitation assay
RPE	Rate of perceived exertion
SC	Satellite cell
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer of transcription 3
TGF-b	Transforming growth factor beta
VEGF	Vascular endothelial growth factor
VO_2max	Maximal oxygen capacity
V_T	Ventilatory threshold
WGA	Wheat germ agglutinin

List of figures

List of tables

Chapter 1: Introduction

Skeletal muscle is one of the most dynamic tissues in the human body. It has the ability to adapt to its local environment in response to external stimuli which is evident in, for example, increased muscle function noted in athletes who adapt in specific ways to either a strength training or endurance training regimen and conversely the decreased function of those suffering from various types of muscle disease and atrophy. High intensity training (HIIT) protocols improve muscle function and have been used by athletes in a performance oriented manner (1) and as a medical intervention in a rehabilitation oriented manner (2). The wide use of HIIT is indicative of the importance of understanding the mechanisms behind high intensity training induced muscle adaptation.

HIIT interventions are used popularly by endurance athletes. Training at high intensities may increase muscle strength and increase aerobic capacity at these intensities (98). HIIT running as a protocol has been shown to increase laboratory performance factors such as peak treadmill speed, VO_2max and running economy (3), whilst muscle adaptation studies are extremely few for running HIIT (see literature review chapter) which differs substantially from non-weight-bearing cycling HIIT.

Muscle contractions can be classified into three classes: eccentric, concentric and isometric contractions, where the muscle fibre lengthens, shortens and remains constant, respectively, upon contraction. Running involves the use of both eccentric and concentric contractions, with the level of the running surface determining the ratio between the two. Downhill running is considered eccentric-biased exercise and conversely uphill running is considered concentric-biased exercise. Uphill running and downhill running are therefore two different role players in muscle adaptation, although potentially both act through regulating satellite cell (SC) dynamics.

The SC is the muscle specific stem cell which resides in a quiescent state in its niche until activation by an external stimulus, which alters some property or properties of the niche (108). These cells are able to proliferate rapidly and ultimately regenerate the fibre after

injury. Their role in exercise induced muscle adaptation is also of great interest to researchers (4). Understanding how this cell type reacts to different types of HIIT and the mechanisms at play will allow athletes and coaches to optimize muscle adaptation for peak performance and will also help doctors and researchers design appropriate therapeutic HIIT for muscle diseases and atrophy.

The microvascular system of skeletal muscle is a capillary network comprised of endothelial cells arranged as a tubular network. The capillaries are responsible for delivering oxygen and nutrients to the muscle and removing waste from the muscle environment. Similar to SCs, endothelial cells proliferate in response to an external stimulus and form a larger capillary network for greater muscle perfusion (226.). Increased muscle perfusion could enhance the capillary function leading to an increase in muscle performance.

The research study in this thesis investigated muscle adaptation in response to two HIIT protocols, namely uphill and downhill running over a 4-week period. Specifically, the SC response and vascularisation was assessed. Chapter 2 presents a review of the literature of skeletal muscle and its adaptation to exercise in general and specifically in response to running. The assessment of the literature on adaptation will primarily be viewed from the point of the SC and the vasculature and the possible increased performance and muscle function as a result of adaptation to these. As this thesis research utilized an *in vivo* model, an attempt was made to focus the reviewed literature on that available for human models. In some instances research data on the particular process are not available. Cell culture and animal models were reviewed in those cases. Chapter 3 entails the aims, objectives and hypothesis for the thesis research. Chapter 4 presents a detailed description of the methods and materials used to complete this research. Chapter 5 will be the results section and chapter 6 the discussion thereof. Chapter 7 will entail concluding remarks with chapter 8 bringing the thesis to an end with a brief discussion on areas of improvement and future recommendations.

Chapter 2: Literature review

2.1 Skeletal muscle

Skeletal muscle comprises of roughly 40% of body mass and is one of the most dynamic tissues in the body, with the ability to adapt to external stimuli placed on it. It functions on a mechanical level, responsible for locomotion and posture maintenance by generating force to operate the lever system of the skeleton (5). Skeletal muscle has multiple physiological functions. On a metabolic level skeletal muscle functions as storage for metabolic substrates for its own use or for mobilisation and contributes to basal metabolism. Roughly 80% of glycogen is stored within skeletal muscle (6) and in fasting states skeletal muscle breaks down proteins into amino acids for energy regulation. It functions in the maintenance of thermoregulation by producing heat contributing to core temperature (7). Structurally, skeletal muscle fibres are multinucleated with a complex intracellular protein network responsible for muscle contraction, structural integrity and cell signalling. A variety of proteins situated on the periphery of muscle fibres make up the extra-cellular matrix. There are multiple cell types within skeletal muscle that include immune cells, the vasculature fibroblasts and resident stem cells known as Satellite cells (SCs) to name a few. Athletic performance is largely reliant on optimal skeletal muscle health and function as well as its specific adaptions to training interventions. Understanding muscle function and regeneration will not only allow athletes to optimize their performance but also improve quality of life of the general public. This review will explain the structural components of skeletal muscle and the interplay with other cell types and stimuli at the physiological level and in response to exercise training.

The structural make up of skeletal muscle consists of the muscle fibres held together in the perimysium. The contractile unit of skeletal muscle is the sarcomere, which is roughly 2 µm long. An understanding of the basic structure of the sarcomere is important for an understanding of different types of muscle contractions. The structural integrity of the sarcomere is held together by the thick filament myosin, 215 kDa, (8) the thin filament actin, 42 kDa ,and the titin filament, 3.0-3.7 MDa (9, 10). The boarders of the sarcomere are known as the Z-discs which connect the thin filaments of two adjacent sarcomeres. The A-band consists of thick filaments and portions of thick filaments which overlap with the thin filaments. In the middle of the A band is an area where no thin filaments are present, known as the H-zone, and in the middle of the H-zone and the sarcomere is the M-line.

The I-Band is the area which only consists of the remaining portion of the thin filaments (Figure 1). One sarcomere consists of 1 full A band and 2 half I-bands. Titin is a giant protein which extends from the Z-disc, where its NH2 terminal is connected to the protein nebulin, to the M line, where its COOH terminus is connected to the thick filament myosin (9).

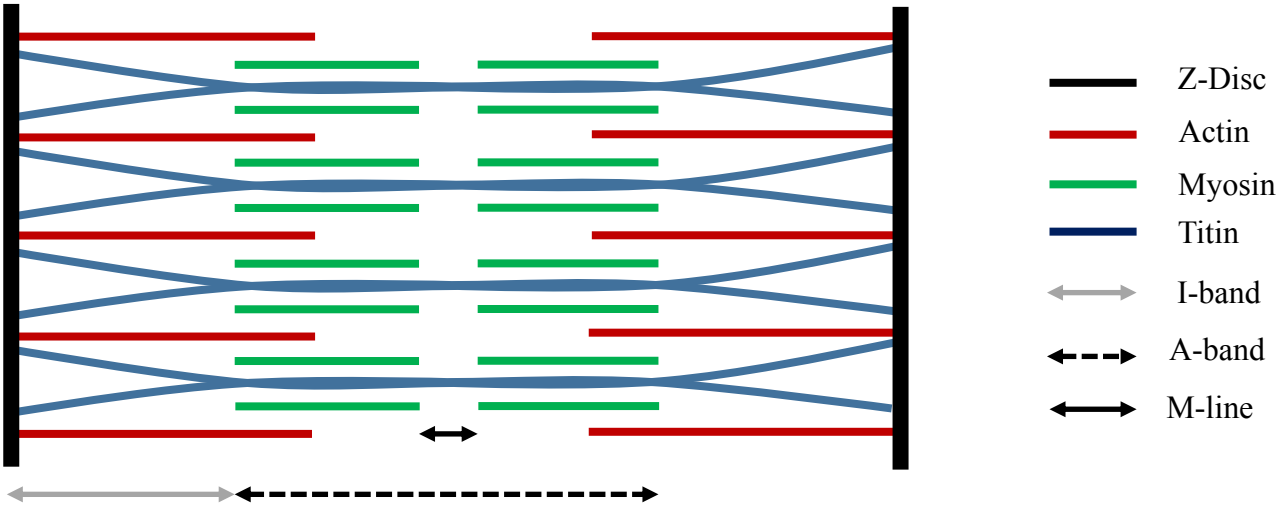


Figure 1. Representation of the muscle sarcomere and the contractile proteins of skeletal muscle, actin, myosin and titin.
One sarcomere extends from Z-disc to Z-disc. Actin and titin are anchored to the Z-disc via anchorage proteins. Regions of the sarcomere are divided into areas according to the presence of certain contractile proteins. The area where only actin and titin are present is known as the I-band, the area where only myosin and titin are present is known as the A-band and the area where neither actin nor myosin are present is known as the M-line.

Muscle contractions are either classed as isotonic, where the sarcomere length is altered with contraction because the tension produced by the muscle is different to the load placed on the muscle, or isometric, where sarcomere length is constant because the tension produced by the muscle is equal to the load placed on the muscle. There are two types of isotonic contractions, concentric contractions where the sarcomere length shortens with contraction and eccentric contractions where the sarcomere lengthens with contraction. The traditional theory of muscle contraction is the sliding filament theory which involves sarcoplasmic reticulum release in calcium and subsequent binding of actin and myosin forming cross bridges with one another, the power stroke of the myosin heavy chain and shortening of the sarcomere (11). This theory doesn't consider eccentric contractions, where actin and myosin cross bridges are broken due to the excessive load placed on the muscle. A greater force is generated by the muscle under eccentric contraction compared to concentric contraction. This could be due to the cross bridge requiring a greater force to break than what it produces in a concentric contraction. Interestingly enough, in muscle fibres which are stretched beyond

actin and myosin interaction (sarcomere length of 4µm), one would expect a loss in muscle force production. However, at this point there is a 200% increase in force production (12). Titin is therefore proposed to be the mechanism of action for this spike in force, whereby the titin filament PEVK domain is tightly wound together in a spring like formation and unwinds at lengths where actin myosin interactions are lost. This is known as the winding filament theory and could explain why eccentric contractions produce more force than concentric contractions (12).

Skeletal muscle fibres are distributed between two main fibre types, type I and II and two subtypes of type II fibres according to what iso-form of myosin heavy chain (MHC) they possess (13). Type I fibres, known as red fibres, possess the iso-form MHC-I. They are characterized by a large mitochondrial content and oxidative enzymes, they are small in size and thus have a low contraction force but don't fatigue easily. Type IIx fibres on the other hand are known as white glycolytic fibres, they have fewer mitochondria than type I fibres, and are larger in size, thus producing a more forceful contraction, yet they fatigue easily. Type IIa fibres are characterized as glycolytic fibres with a high mitochondrial content, as they share characteristics of both type I and type IIx fibres. Hybrid fibres are fibres that have a combination of MHC iso-forms IIa/x (14). These fibres are able to transition into one or the other iso-form they possess and become a pure fibre. The average sedentary individual will have an even distribution of fibre types (15). With extensive exercise training there is a shift in fibre type distribution according to the nature of the training, endurance training will cause a shift towards type I whilst power athletes such as sprinters, jumpers, and power lifters will have a shift towards type II with a distribution between types IIa and IIx depending on training factors that might also influence speed endurance (16).

2.2 Concentric and eccentric exercise

Exercise movements typically cycle between eccentric and concentric contractions. It has been described that the different contraction forms cause specific muscular responses and therefore could lead to different training adaptations (17). Due to the constant cycling between concentric and eccentric contractions with movement, exercises are considered concentrically biased if they involve predominantly concentric contractions for force

production. The opposite predominance of contraction type denotes eccentrically biased exercises.

It has been well established in the literature that eccentric exercise causes muscle damage. It was first described in 1902 by Hough, when he described muscle pain after eccentric bouts of exercise. The symptoms were described to occur 24-48 hours after the exercise bout and was termed Delayed Onset of Muscle Soreness (DOMS), now a commonly known term (18).

The mechanism was not described but it was suggested that the soreness was due to micro-tears in the muscle. The first structural evidence associated with DOMS was reported by Friden et al after a bout of downstairs running. Electron microscopy showed disrupted sarcomere integrity, identified by a non-uniform alignment of the Z-discs of adjacent fibres, known as Z-disc streaming (19). Also, an acute bout of eccentric exercise has been shown to cause swelling of the myofiber, infiltration of inflammatory cells and damage to the myofiber membrane shown by a loss of dystrophin staining (20). A study comparing the acute effect of maximal eccentric vs concentric knee extensions showed an increase in muscle protein synthesis 8 hours after exercise in the eccentric group (21), which was interpreted as a repair response to replace damaged proteins. Although eccentric exercise is well correlated with muscle damage, unaccustomed concentric contractions have also been shown to cause muscle damage (22 23). A study by Gibala et al., consisted of subjects performing the eccentric phase of a bicep curl with one arm and the concentric phase with the other arm. Muscle samples were taken and sarcomere disruption was noted via electron microscopy in subjects in both arms 48 hours post arm resistance exercise, suggesting muscle damage may be caused by both the concentric and eccentric phase of weight lifting (24). The results from acute studies support the phenomenon of the destructive nature of eccentric exercise when muscle is unaccustomed to this stimulus, resulting in muscle damage, an inflammatory response to clean up the cellular debris and assist in the regeneration process, and the subsequent muscle protein synthesis in order to re-establish muscle function. These acute studies are important to understand the molecular and morphological responses to muscle damage but do not address potential muscular adaptation or myofibrillar remodelling from repeated eccentric or concentric exercises.

2.3 Exercise training

Resistance training has been used to increase muscle mass, strength and function for decades (25, 26). The style of training, specifically, may elicit different strength gains but both eccentric and concentric training have resulted in an increase in muscle strength (27). See table 2.1 which illustrates the exercise type, basic details of the subjects, study design, and main findings of either a concentric or eccentric exercise training protocol or a combination of both on muscle strength. Eccentric training has the capacity to generate increased muscle strength at a lower metabolic cost compared to concentric contraction, making it a 'promising' training intervention (28). This difference points to the possibility that the adaptations may be specific in a metabolic sense. Additionally, eccentric training has been reported to maintain muscular strength and endurance, after a period of detraining, more effectively than concentric training (29). This finding has been confirmed by another study by Coratella et al (2016) (30), details of the study can be found in Table 2.1. Nonetheless, both contraction forms are essential to muscle function in everyday life activities and in sport performance, with many activities requiring a complex interaction between the two.

The stretch shortening cycle is a biomechanical phenomenon which explains energy storage in the eccentric phase of exercise in order to produce a more powerful concentric contraction. The basis of this exercise type is split up into three phases. Phase 1: the loading phase, where the muscle contracts eccentrically, loading kinetic energy as it does so. Phase 2: Transition phase, where the muscle contraction changes from an eccentric contraction to a concentric contraction. Phase 3: Explosive phase, where the stored kinetic energy that the muscle has built up during phase 1 is transferred into the concentric contraction, producing a powerful contraction. The most commonly used example to explain the stretch shortening cycle, is comparing a standing jump with no eccentric loading phase to a squat jump where the squat movement eccentrically loads the muscle and the outcome is a greater maximum jump height (31).

The stretch shortening cycle is also used during running since a runner cycles between eccentric and concentric contractions with every stride, and is considered an efficient and energy saving form of locomotion (32). Downhill running evokes an enhanced stretch shortening cycle compared to level surface running (33). Eccentric training has been used by coaches and physiotherapists/rehabilitation specialists to increase muscle strength and power

output in the concentric phase of a movement (34). The mechanisms of adaptation to training will provide coaches, doctors, physiotherapists and other individuals involved in improving muscle function of an individual with insight to understand the efficacy of a given exercise protocol. See table 2.1 for a summary of the how the contraction type may influence muscle strength gains in response to resistance training protocols. What can be noted is that training periods in the studies presented here ranged from 4-10 weeks and involved various training modes such as an isokinetic dynamometer, a motorized treadmill and bench press apparatus. Both contraction forms may increase muscle strength after a prolonged resistance training period.

Table 2.1: The effect of contraction type during resistance training protocols on muscle strength

Exercise mode	Subjects	Exercise type	Intervention	Equipment	Muscle strength	Main finding/ Conclusion	Reference
Eccentric	Healthy young untrained females	Eccentric or concentric only	10 weeks 3 sets 10 reps 3 times per week	Isokinetic dynamometer	↑ Concentric group ↑ Eccentric group	Muscle strength was increased in a contraction specific manor	(35)
Eccentric vs concentric	Moderately trained young males	Eccentric or concentric only	6 weeks 4 sets 6-8 reps 3 times per week	Isokinetic dynamometer	↑ Concentric group ↑ Eccentric group	No difference in peak knee flexion torque between groups	(27)
Eccentric	Healthy young active males	Downhill running	5 weeks 20 mins at LT 3 times per week	Motorized treadmill	↑ Eccentric group	Downhill running increased peak knee flexion torque	(36)
Eccentric vs concentric	Healthy young active males	Eccentric or concentric RT	6 weeks RT and 6 weeks detraining	Bench press apparatus	↑ Concentric group ↑ Eccentric group	Eccentric group maintained strength and muscle endurance after detraining	(30)
Eccentric-concentric	Patients with elbow flexor tendinopathy	Eccentric or eccentric and concentric elbow flexor RT	4 weeks Contraction based rehab exercises 5 times per week	None	↑ Eccentric -concentric group ↑ Eccentric group	Both eccentric training and eccentric-concentric training improved function	(29)

RT Resistance training LT Lactate threshold

2.3.1 Adaptation to muscle damage

As mentioned above, eccentric exercise induces muscle damage and regeneration. It has been noted that subsequent eccentric bouts do not induce muscle damage of the same nature due to an underlying protective effect, this phenomenon is known as the repeated bout effect (37). Initial findings of the repeated bout effect by Byrnes et al., used downhill running as a model for eccentric exercise induced muscle damage. Subjects were randomized into 3 groups performing 2 exercise bouts separated by 3, 6 and 9 weeks of no activity respectively for each group. Perceived muscle soreness after the 1st bout of downhill running was elevated as expected. Also, creatine kinase (CK), an enzyme found in high quantities in skeletal muscle and usually in low quantities in circulation, was elevated in blood samples. The second exercise testing session took place at either 3, 6 or 9 weeks after the 1st bout. The perceived muscle soreness and CK response after bouts at 3 and 6 weeks were blunted compared to the 1st bout, whereas the subjects who did their second bout 9 weeks after the 1st bout showed a similar response to the 1st bout (38). Attempts have been made to understand the mechanism of the repeated bout effect, which are summarised into three mechanisms of adaptation.

2.3.1.1 Neural Adaptation

A un-accustomed bout of eccentric exercise caused muscle damage, specifically in type II fibres (39, 40). The neural adaptation theory suggests that motor unit recruitment during the following exercise bout, is increased, the expansion includes type I fibres which then will allow force to be distributed over more fibres (41).

2.3.1.2 Non-contractile protein adaptation

Eccentric exercise places strain on contractile proteins of skeletal muscle but also on non-contractile proteins and tissue. These proteins include the extracellular matrix (ECM) and the intermediate filaments (IF) which function as a scaffold, responsible for a substantial contribution to the maintenance of structural integrity of the myofiber (42) and force transmission (43, 44). Select regions of some of these components also act as signal transducers to initiate gene transcription responses (45). Three days following eccentric exercise there is increased transforming growth factor-beta (TGF-b) signalling and collagen expression, suggesting structural remodelling (46). IFs may be damaged and re-organized during the regeneration phase; the combination of both may allow for more effective force transmission in future eccentric bouts, with less muscle damage as a result (41).

2.3.1.3 Muscle Cell adaptation

Eccentric exercise causes sarcomere disruption, with some sarcomeres returning to normal lengths and others remaining stretched following exercise (47). In instances when there hasn't been adaptation to motor unit recruitment properties of skeletal muscle following an eccentric bout, an alternative proposal has been that adaptation may occur at the muscle fibre or sarcomere level (48). The sarcolemma is defined as the muscle fibre cell membrane. Strengthening of the sarcolemma may prevent its rupture during a second bout of eccentric exercise and reduce excessive calcium efflux from the sarcoplasmic reticulum, which is linked to the sarcolemma by the T-tubule network, thus reducing calcium-induced cellular degradative processes.

2.3.2 Biomarkers of muscle damage

Creatine kinase (CK) is the enzyme used in the reversible conversion of phosphocreatine and ADP to ATP which is the product generated by the reaction. It is used as a marker of muscle damage as muscle damage causes ruptures to the sarcolemma allowing CK to escape and enter circulation. Following eccentric exercise there is an increase of CK in the circulation. Whether or not the increase in circulating CK can be tightly correlated with the perceived muscle pain and DOMS is controversial, with Coratella et al. (2016) providing evidence in support (49), but not according to Chimera et al. (50). Following eccentric exercise there is a peak in serum CK activity levels which frequently occurs at 96 hours post exercise (49) whilst the peak is 8 hours after strength training (51). CK is commonly used as an indirect marker for muscle damage as it can be easily detected in the blood. The use of CK activity as a muscle damage marker presents difficulties as there are many factors other than muscle damage that cause higher CK levels in circulation. Individuals who train daily will have higher resting CK activity levels (52). Individuals training frequently, even at a recreational level have elevated resting CK, but these elevations subside with sufficient recovery (53). Athletes have higher resting CK activity levels but post exercise the extent of increased serum CK activity levels does not reach the same magnitude of sedentary individuals, most likely due to the fact that the athletes' muscles have adapted and there is less muscle damage (54). There are differences between genders, race (55), predominant muscle group exercised, hydration status, and fibre type distribution of the individual, to name a few (273). Other enzymes that have been proposed as biomarkers for muscle damage are Lactate

dehydrogenase (LDH), the enzyme which catalyses the reaction of pyruvate to lactate (or reverse), and aspartate aminotransferase, the enzyme which catalyses the reaction of aspartate to glutamate (56). It has been proposed that the increased levels of any of these enzymes is the same, namely the loss in sarcolemma integrity (57).

Various proteins other than enzymes have also been identified as biomarkers of muscle damage, the most common of which is myoglobin. Myoglobin, is the haem protein that plays the role of binding oxygen to its iron moiety and with a cascade of binding and release, facilitating oxygen diffusion in skeletal muscle, as opposed to haemoglobin which is responsible for oxygen transport in the circulation (58). Similar to CK, following structural damage to the muscle fibre myoglobin escapes the muscle fibre and enters circulation (59). Troponin is a protein which has 3 different protein complexes (Troponin I, C and T) involved in regulation of skeletal muscle and cardiac muscle contraction and therefore has skeletal and cardiac muscle specific iso-forms (60). Troponin C is upregulated after muscle damaging exercise (61), but also after myocardial damage. Xin is a protein that is predominantly located near the myotendinous junction but has been seen to localise across a whole muscle fibre post injury and during myopathies (62). Following a bout of exercise there was upregulation of Xin in individuals who performed eccentric exercise when compared to a workload-matched concentric group (63), suggesting that Xin is a marker of eccentric muscle damage for healthy untrained individuals.

When conducting research with human participants, there is a large degree of variation between individuals. Responders and non-responders are terms used to identify sub-populations within studies that either respond or don't respond to the intervention. In some instances, when there is no mean change due to an intervention, the individual responses may provide a more detailed perspective on the effect of the intervention. Using the responders and non-responders approach also drives sport science and physiology research to a more individualistic approach, paying more attention to individual needs rather than making conclusions due to mean data. When classifying an individual as a responder or a non-responder one could repeat the tests and measurements to ensure that the individual is a responder or not. However, this is not feasible when it comes to eccentric exercise protocols, due to the second bout effect possibly influencing the categorisation.

To get a full view of muscle damage, it is advised to measure a combination of these muscle damage markers, yet even then they are still indirect measures of muscle damage and don't fully elucidate the extent of muscle damage.

To summarize, eccentric exercise induced muscle damage results in symptoms such as increased muscle damage markers, CK and myoglobin, in circulation as well DOMS, reduced capacity to produce force and loss in range of movement. On the microscopic level, ultrastructural damage can be observed, visualized by Z-disk streaming or damage to the muscle fibre membrane, or both (19, 20). Repeated bouts of exercise induce an adaptive response potentially on multiple levels, one of them being the regeneration of muscle which will be discussed later on, and an increase in muscle functional capacity.

2.4 Running

2.4.1 Running, training and performance factors

The goal of a competitive runner is to run the required distance as quickly as possible, or quicker than the closest competitor (64). Factors that impact performance include running economy, maximal oxygen consumption (VO_{2max}), or the capacity to exercise at high percentages of VO_{2max} (65). Training induces adaptation to the cardiovascular system, the metabolic processes, mechanical efficiency and skeletal muscle, factors which all contribute to running performance. (66).

VO_{2max} is the maximal oxygen consumption, the majority of which is utilized by the muscles at peak sustained workloads and is considered to be an indication of aerobic fitness (67). Young untrained males are estimated to have a VO_{2max} of 48-50 $ml.l.kg^{-1}$ according to a review by Shvartz published in 1990 (68). VO_{2max} will increase with endurance training and correlates well with performance in amateur and untrained individuals. The highest ever recorded VO_{2max} was that of the Olympic cross-country skier, Bjorn Daehlie, with a value of roughly 95 $ml.l.kg^{-1}$ (69). Saltin and Astrand (1967) were the first to compare the maximal oxygen uptake amongst athletes and discovered that cross-country skiers had the highest

VO_{2max} ,roughly 80 - 85 ml.l.kg^{-1} , followed by endurance runners e.g. 800-1500m runners (roughly 78 - 82 ml.l.kg^{-1}) and endurance cyclists (roughly 72 - 80 ml.l.kg^{-1}), with swimmers exhibiting a wider range (roughly 58 – 70 ml.l.kg^{-1}), weight lifters fairly moderate (52 – 58 ml.l.kg^{-1}) and untrained individuals low to very low (38 – 48 ml.l.kg^{-1}) (70).

Testing an individual's VO_{2max} has been a standard procedure in athlete assessment across a variety of sports and levels of participation and is most accurately performed with gas exchange spirometry apparatus. Determining the VO_{2max} of an athlete has been valued by conditioning coaches for quite some time. Protocols have been developed to estimate one's VO_{2max}. Generally, these tests correlate well with measured VO_{2max} (61, 72). Peak treadmill speed on a maximal incremental exercise test correlates well with VO_{2max}. This is advantageous for coaches or trainers who have high speed treadmills but don't have access to gas analysis equipment An individual's VO_{2max} may vary for different exercises and therefore the test result of VO_2 is exercise-specific (73, 74). This becomes an important factor when choosing the type of VO_{2max} test for an individual.

VO_{2max} correlates well with performance for amateur and untrained individuals but correlates less well with performance in elite athletes. Running performance and VO_{2max} rely on central adaptation like increased stroke volume and cardiac output and other peripheral adaptations, like capillary density, but also running economy. VO_{2max} and running economy only partially correlate and therefore when testing, must be determined independently (75). Not only does VO_2max plateau during the final workloads of an incremental test to exhaustion, but improvements will also plateau in well trained athletes, suggesting a limit to this physiological variable. Nevertheless, it has been suggested that anaerobic characteristics such as lactate threshold and anaerobic capacity play an important role in endurance performance (76).

During exercise there is an increase in glycolytic energy provision in the muscle fibres, resulting in the production of pyruvate which is either incorporated into the Krebs cycle, predominantly under aerobic conditions, or converted into lactic acid, either under anaerobic conditions or when mitochondrial content is low (77). In either of these situations, the production of pyruvate exceeds its incorporation into the Krebs cycle and results in an accumulation of intramuscular lactic acid and subsequent lactate in circulation (78). The

circulating lactate levels are influenced not only by appearance (release by exercising muscle) but also disappearance (removal from circulation). The anaerobic threshold is the point of work during exercise where lactate production and clearance are still relatively equal and subsequent increase in exercise intensity will cause an exponential increase in blood lactate (79).

If there is an even higher accumulation of lactate in circulation during exercise to the extent that it cannot be effectively removed, it may be assumed that the individual is exercising at a point higher than the lactate threshold (80). Measuring an individual's lactate threshold is proposed to be an indirect measurement of aerobic capacity (81), and has traditionally been achieved by measuring the lactate quantity in circulation to determine a turning point where the increase in lactate with increase in workload is no longer linear. L_T is expressed as the intensity of exercise associated with the above-explained accumulation of lactate in circulation during an exercise test and is used often used to set training intensities by using the velocity or work associated with the lactate threshold (L_T) (82). Similarly, as the work required increases, breathing rate increases in a linear fashion. The V_T is the point during an incremental exercise test when there is a break in ventilation (82). In the absence of blood sampling to determine the (L_T), ventilatory data may be used to determine the ventilation threshold (V_T) (82). Therefore, V_T is a less invasive method to determine the so-called anaerobic threshold. Training may delay the onset of the ventilatory break point. Training will delay the onset of the L_T and V_T but will also improve an individual's capacity to maintain work for sustained time after the onset of the L_T and V_T (82). Not all sport scientists are satisfied with L_T and V_T assessed during incremental workload tests as determinants of the anaerobic threshold and attempts to define the anaerobic threshold include a variety of exercise protocols and lactate measurements (83), but discussion of these is not within the scope of this review.

2.4.2 Running economy and oxygen cost of running

The goal of runners is to move as efficiently as possible, expending as little energy whilst still performing at high levels. The energy cost of running is defined as the amount of energy needed to transport a body from one point to another and running economy has been referred to as the steady-state VO_2 at submaximal velocity. Strategies to improve running economy include, endurance training, resistance training, neuromuscular training, training in different environments and nutrition (64).

2.4.3 Eccentric and concentric running

Running requires both eccentric and concentric contraction forms. On a level running surface the ratio between the two contractions is fairly equal, but as the gradient profile changes the ratio between the two contraction forms are altered. Downhill running shifts the ratio to favour eccentric contraction, and thus is termed an eccentrically biased exercise, whereas uphill running shifts the ratio to favour concentric contraction and thus is termed a concentrically biased exercise. As explained before, downhill running was initially at the forefront of eccentric exercise induced muscle damage studies (19). When compared to level surface running, there was an increase in DOMS and CK at 24 hours after exercise due to the sarcolemmal damage caused by the mechanical stress of downhill running in the downhill group but not in a work matched level surface group (84). Uphill running is considered as a concentrically biased exercise for the quadriceps muscles. When comparing the muscle damage between an acute bout of uphill and downhill running, there was an increase in CK levels 24 hours post exercise in the downhill group but not the uphill group. (85). The mechanical stress of downhill running is greater than that of uphill running and level surface running. When comparing uphill running to downhill running and level surface running, at constant velocity, uphill running requires more oxygen utilisation, there is a higher concentration of blood lactate and a lower plasma pH (86).

A downhill training program lasting 5 weeks, with each session consisting of 20 minutes of running at velocities associated with L_T, resulted in no improvement on aerobic capacity (36). Uphill running therefore is considered to have a higher metabolic overload than that of downhill and level running. Uphill running requires the muscle to produce more forceful concentric contractions because the overall movement direction is opposing gravity. The

muscle then needs to increase metabolism to provide ATP to the muscle. ATP is needed to bind to the myosin head and break the cross-bridge between actin and myosin during contraction, and to be hydrolysed to provide energy for the following myosin head power stroke. Downhill running also requires ATP for cross-bridge cycling, but the nature of the force placed on the muscle during eccentric contraction will break the cross-bridge and the energetics therefore differs. At some stage of continuous uphill running the oxygen requirements will supersede the oxygen supply to the working muscles and metabolism will shift predominantly from aerobic oxygen metabolism to anaerobic metabolism. An increase in lactate accumulation in the circulation will be a result of this.

2.4.4 Skeletal muscle adaptation to running

The increase in VO_{2max} and aerobic capacity due to training has been proposed to be a result of central cardiac adaptation, namely an increased stroke volume and cardiac output, allowing more efficient O_2 and nutrient delivery via the larger arteries to the muscle. Vascular adaptation in the periphery allows for better delivery via the microcirculation and for an improved venous return, allowing for more efficient waste removal and transportation of waste to be metabolized e.g. by other muscle or liver. Skeletal muscle adaptations occur before central adaptations. Such adaptations occur with training and they include an increase in VO_{2max}, mitochondrial density, (87), oxidative enzyme activity (88) and capillary density (89). Prolonged aerobic training may alter the fibre type distribution of an individual. Trappe et al. (1997) performed a 20 year follow up study and noticed that trained individuals had a higher percentage of type I fibres compared to that untrained individuals (90).

2.4.5 Training load

Training load usually includes variables such as training frequency i.e. the number of sessions per week or within the micro-cycle of multiple weeks, and the training volume expressed as hours per week or distance per week and the intensities of the different sessions. The intensity is an interesting debate in sport science, with questions arising about what is more beneficial for the athlete and the performance: high intensity low volume or low intensity high volume?

2.5 High Intensity Interval Training

High intensity interval training (HIIT) doesn't have a universal definition, but in short it is exercise sessions which consist of short to long intervals of work with rest periods between intervals, at an intensity which is near maximal Heart rate (HR) and VO_2. This definition assumes that one or the other of these physiological variables is measured, which may not always be the case. Nevertheless, the main characterisation of HIIT is that its intensity is so high that the duration of each interval cannot be sustained for a long period of time and together this results in a low volume for the session. HIIT has been studied from the point of view of its influence on performance parameters and in a physiological context from the point of view of lactate production, submaximal oxygen consumption and the heart rate response. Although substantially less, there is some research that has been done on potential muscular adaptations. The first performance increases due to HIIT were reported in 1977 when firefighters underwent a 10-week running and circuit HIIT program (91). Some of the initial muscle research involving HIIT analysed the glycolytic enzyme activity alterations due to training (92).

More recently the intramuscular effect of HIIT has been compared to that of moderate intensity continuous training utilising cycling as the mode of exercise. A recent study was done by Fiorenza et al. (2018) and the results showed that HIIT promoted mitochondrial biosynthesis. Compared to work matched moderate intensity continuous cycling and high intensity repeated sprint cycling (which typically has even shorter duration and higher intensity than HIIT), high intensity speed endurance cycling increased phosphorylation of p38 MAPK and CaMKII, and an increased mRNA response of PGC-1α and HSP72. This suggests that despite the reduced volume the metabolic response to high intensity exercise promotes mitochondrial biogenesis (93). This is supported by MacDougall et al. (1998) who administered a repeated Wingate sprint training protocol which resulted in increase in glycolytic and oxidative enzyme activity as well as VO_{2max} (94). When comparing a 2 week low volume repeated sprint training intervention to traditional high-volume low intensity aerobic training in cyclists, it was found that there was no difference in performance improvements, nor the oxidative capacity improvement of the muscle when comparing muscle biopsies from before and after training (95). The same study demonstrated that HIIT is a more time efficient training modality compared to traditional aerobic training, as the

groups exercised for a total of 15 min and 630 min respectively yet obtained similar adaptation (95). Therefore, one of the main advantages of HIIT is that it is time effective. Exercising at near maximal intensities results in an overall session time of between 20-60 minutes (95). The incorporation of HIIT into the exercise programme of recreational triathletes, decreased the weekly running load from an average of 33,6 km to 10,1 km whilst still increasing sprint distance triathlon performance (96). Other studies have also reported similar performance and physiological improvements when comparing HIIT and traditional endurance training and the time advantage that HIIT offers (97).

One must take caution when reviewing literature on HIIT, due to the variation in exercise interventions used, exercise modality, duration and intensity of session, and the ratio between work and rest that the protocol may demand. From this point, the focus will be running HIIT studies, specifically low volume high intensity protocols.

2.5.1 Performance improvement due to running HIIT

Key parameters to change with training that are known to improve running performance involve those that improve VO_2max and muscle power output (94). HIIT has been used to improve cardiovascular fitness by enhancing VO_2max and O_2 pulse, post training (98). A study performed by Billat et al. (2003) showed an improvement in 10 km performance in Kenyan professional long distance runners, after a HIIT intervention (99). Yet results are still conflicting, since HIIT resulted in no improvement in VO_{2max} and peak power output compared to a steady state aerobic training protocol. This particular study showed that not only is HIIT not superior to traditional aerobic protocols but subject feedback from a questionnaire described HIIT as less enjoyable than the traditional aerobic exercise intervention (100). The lack of enjoyment could be due to the fact that the study participants were untrained individuals who are unaccustomed to the nature and intensity of HIIT. It is not surprising that a bout of exercise at high intensities produces a low score on an enjoyment spectrum, but studies that use this form of assessment must ensure that the study design incorporates a training program. This may allow for adaptation to the training and potentially more enjoyment during the exercise session. In summary, HIIT may be of benefit in obtaining improved performance and physiological adaptation that is sought after by athletes.

In the literature, the possibility that HIIT may attenuate the detrimental side effects from disease or pathological states has been a more recent topic.

HIIT has been used either in isolation or as a concurrent exercise intervention with resistance training to combat diseases such as sarcopenia (101), multiple sclerosis (102), coronary heart disease (103) and type 2 diabetes (104), to name a few. The beneficial effects of HIIT have also been noted in the rehabilitation of patients who have suffered an ischemic episode such as a myocardial infarction or a stroke (105, 106). In this context, HIIT has been seen to be as a superior exercise intervention compared with moderate-intensity continuous training with better improvements in maximal oxygen uptake (105). The nature of HIIT ensures the exercising individual works at near maximal intensities. The longer duration spent at near VO_2max during training sessions could explain the superior cardiovascular adaptation compared to the moderate intensity exercise group.

In the literature reviewed above, general cardiovascular fitness and performance factors were measured to assess HIIT induced adaptation. In the context of adaptation to training, intramuscular adaptations may play a pivotal role and may also be regenerative in nature.

2.6 Satellite cells

Skeletal muscle's regeneration capacity makes it a unique tissue offering big insights into regenerative engineering. This is for two reasons. Firstly, each muscle cell is multinucleated, but these nuclei are post-mitotic. Secondly, during the development of skeletal muscle, not all the nuclei are differentiated into myonuclei, but a select pool of nuclei does not follow the same fate and stay in a position to become a myogenic stem cell, known as the satellite cell (SC). The SC resides in its niche with is located between the sarcolemma and the basal lamina of a skeletal muscle fibre (107), where it is able to localize according to certain stimuli such as a stable extracellular matrix.

2.6.1 Identification:

Initial identification of SCs was achieved using electron microscopy by Alexander Mauro in 1961 (107), who assessed the specialized localization. The initial observation was that a nucleus relatively larger than a myonucleus was situated between the sarcolemma and basal lamina. The identification of SCs experimentally in more modern times may be achieved by identifying certain transcription factors or membrane proteins specific to SC and no other myogenic cells. The difficulty arises as SCs express varying levels of multiple transcription factors according to their temporal kinetics (108). One of the most commonly used transcription factors to identify SCs, regardless of activation status, is Paired Box (Pax)-7. Pax7 is expressed in quiescent, activated and proliferating SCs *in vitro* and *in vivo*. The expression of Pax7 decreases upon SC differentiation into a myoblast. Other identifiers of SCs have also been in vivo by staining for membrane proteins, Neuronal Cell Adhesion Molecule (NCAM or CD56) (109), M-cadherin (110) and α7-Integrin (108). Identification of SCs has also been achieved with myogenic regulatory factors Myf5 and MyoD, however the limitation to using these myogenic regulatory factors is that they are not expressed in quiescent SCs. Therefore, they may be useful to assess the dynamic state of some SCs but may not identify the total pool.

2.6.2 Satellite cell content in skeletal muscle

SCs reside in their niche in the quiescent state but are easily activated and hence could be described as a static patrol system of the muscle fibre for injuries. Establishing whether or not a SC is truly quiescent is complicated and therefore, 'resting' may be an appropriate descriptor. Generally speaking the resting SC pool size is roughly one SC per ten myofibres in healthy young males and females when assessed in cross-sections of muscle biopsies (see table 2.2). The SC pool is conserved over various muscle types, such as the *deltoid, gastrocnemius, soleus, trapezius* and *vastus laterali,* muscles (see table 2.2). The literature doesn't show any differences between males and females in terms of healthy untrained SC pool size (111, 113). The SC pool size is not fixed and will change according to the stimulus placed on it.

Trained college wrestlers have a larger SC pool size than age and health matched control subjects (111). This increase in the SC pool size is due to training and has been noted in another study where healthy sedentary young males undergo a resistance training intervention (114). The SC pool size may also drop to below healthy resting levels in situations of muscular disease. Sarcopenia is age-induced muscle atrophy and is associated with loss in muscle cross sectional area (CSA) and SC pool size, specifically in type II muscle fibres (115). Exercise training may alleviate the SC loss in older individuals. Life-long endurance runners have smaller type II CSA and SC content than young healthy individuals but have an increased type I specific SC pool as a consequence of fibre type recruitment, unlike untrained older individuals (116). A decreased SC pool size has been seen in patients with *trapezius* myalgia (108) and spinal cord injuries (117). These two conditions differ substantially, since in the former SC may be reduced due to unusual and constant recruitment for differentiation and fusion, whereas in the latter there may be no demand. Unloading following a training protocol decreases the SC pool size back to healthy resting levels (118). The changes in SC pool size as a result of certain stimuli provides insight into the dynamic capability of SCs.

Table 2.2: The satellite cell pool size in different types of human subjects and different muscles

Gender	Subject description	Muscle	SC Pool Size SC/Fibre	Paper
Male	Young sedentary	*Vastus Lateralis*	0.10	(114)
Male	Middle aged sedentary	*Vastus Lateralis*	0.13	(119)
	Middle aged 14 days bed rest		0.08	
Male	Old sedentary	*Vastus Lateralis*	0.07	(117)
Male	College Wrestlers	*Deltoid*	0.10	(111)
	Healthy university students		0.03	
Female	Young, diagnosed with Myalgia	*Trapezius*	0.08	(112)
			0.10	
			0.07	

2.6.3 Satellite cell and regeneration

Following muscle injury, regardless of cause, normally healthy skeletal muscle undergoes regeneration. There is a tight interplay between the inflammatory response to injury and the muscle regeneration (120, 121, 122) as there needs to be a balance between the pro-inflammatory and anti-inflammatory response. The process of muscle regeneration can be separated into 2 phases; the degeneration phase and the regeneration phase (123).

The degeneration phase:

The first response to muscle damage is a pro-inflammatory response. Soon after muscle damage, neutrophils migrate and infiltrate the damaged area in order to engulf and digest damaged muscle fragments (124). After 48 hours, the prominent inflammatory cell type present in the damaged area is the macrophage class that has pro-inflammatory properties, which follows on from the role of the neutrophil (125). In addition to phagocytosis, the second function of the macrophage is to activate the resident SCs by releasing factors such as TNF-α, LIF and IL-6 (126). In order for SC activation to be effective, activation occurs in multiple ways. The immune cells release cytokines, which directly activate the SCs but also

activate other immune cells to release cytokines. Activated SCs, secrete growth factors which activate surrounding SCs in a paracrine fashion, but may also act upon themselves in an autocrine fashion. Growth factors such as IGF-I and II, HGF, and FGF use the vasculature as a mode of transport through the muscle and hence more distant SCs may also become activated (127).

The regeneration phase:

Once the quiescent SCs are sufficiently activated they enter the cell cycle and undergo proliferation. SC activation is not conserved only to the damaged area, other SCs along the myofiber may be activated and migrate to the injured area where they proliferate in accordance with the dominant signals in that niche (108). With severe injury the majority of the active SCs will undergo rapid proliferation. This expanding SC pool will differentiate, align and fuse with existing fibres, or generate new myofibres (118). This sequential process, known as myogenesis, is regulated by the myogenic regulatory factors (MRFs) Myf5, MyoD, Myogenin and MRF4 (127) (Figure 2). Different expressions of the MRFs will cause the SC to enter a specific phase of the cell cycle.

Quiescent SCs express Pax7. Once activated, a small number of SCs downregulate Pax7 expression immediately and only express MyoD and differentiate immediately (Figure 2), most likely to be the initial SCs to contribute to regeneration (115). However, the majority of the SCs maintain expression of Pax7 and upregulate the expression of MyoD and Myf5 and continue to proliferate rapidly (128). mRNA for MyoD and Myf5 are upregulated between 4 and 24 hours (129) and protein levels are upregulated at similar time points (128). Both Myf5 and MyoD bind to the same sites on promotor regions of key muscle genes but MyoD results in gene transcription (130), thus MyoD has been termed as “the master regulator” of myogenesis, as it promotes SC progression to terminal differentiation. SCs that co-express MyoD and Myf5 are destined to become myoblasts, yet there is a sub population of proliferating SCs which maintain Pax7 expression and upregulate Myf5 but not MyoD. This population of SCs will eventually down-regulate the expression of Myf5 and return to quiescence with Pax7 expression only (Figure 2) (108), maintaining the SC pool. As proliferation continues, SCs that begin to lower the expression of both Myf5 and Pax7 shift into a state of differentiation, becoming committed SCs expressing MyoD, myogenin and MRF4 (131) whilst other SCs along the way keep their expression of Pax7, lower the

expression of MyoD and don't express myogenin. These cells exit myogenesis and return to quiescence (Figure 2). Committed SCs lower the expression of MyoD, only expressing myogenin and MRF4 and are committed to terminal differentiation (131). Finally, these cells fuse into existing myofibres or form new myofibres, terminating the myogenic process.

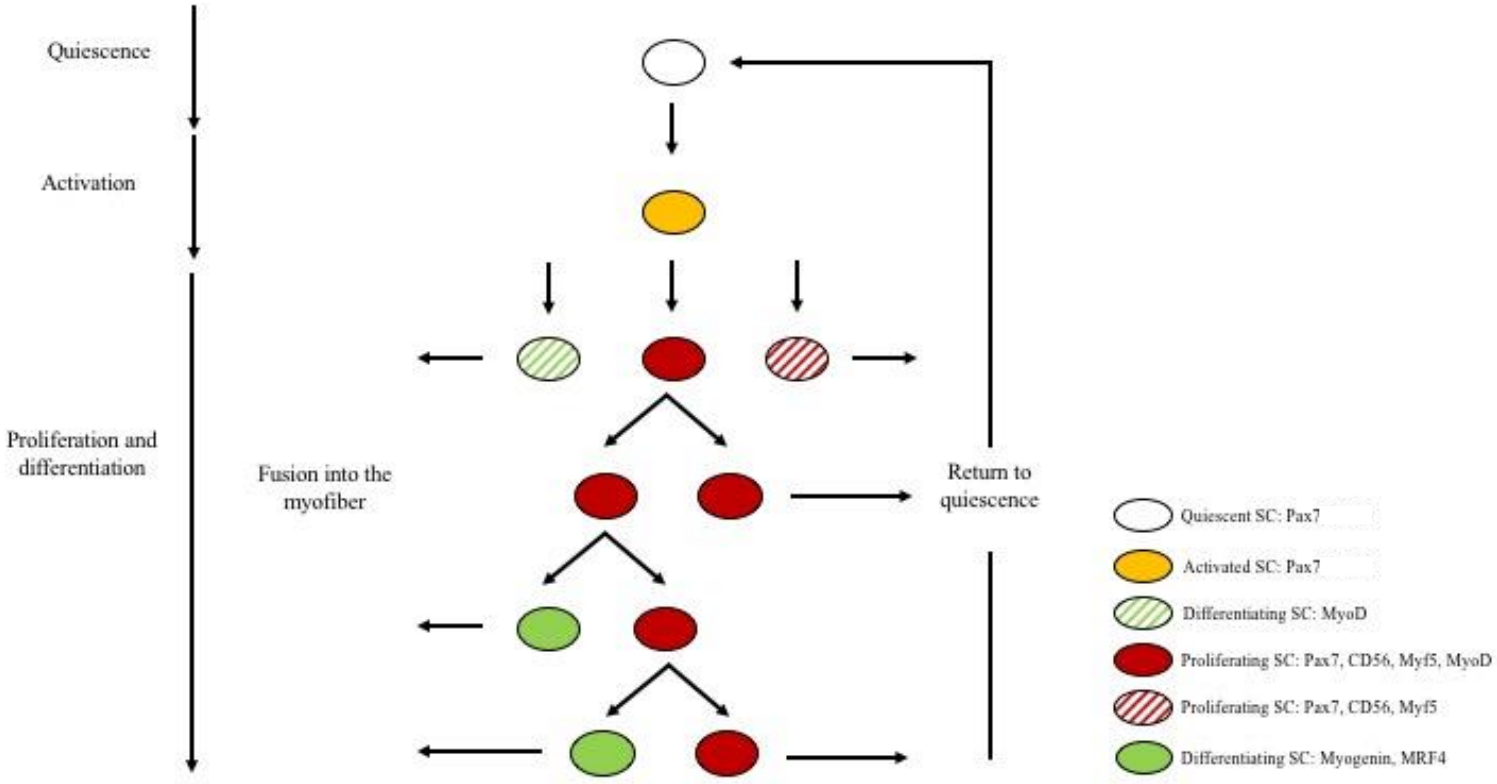


Figure 2: Schematic representation of the myogenic response of SCs and their potential lineage

SCs remain quiescent expressing, Pax7 until activation due to an external stimulus. Upon activation there is an upregulation in the MRFs MyoD and Myf5. Cells which only express MyoD differentiate immediately, cells that upregulate Myf5 more than MyoD are able to proliferate and escape myogenesis returning to quiescence, whilst most cells up regulate both MyoD and Myf5 and begin proliferating with delayed differentiation. Proliferating cells will eventually upregulate the expression of Myogenin and MRF4 and lower the expression of Pax7 and MyoD, terminally differentiating into myoblasts and fusing into the muscle fibre.

2.6.4 Growth Factors and cytokines affecting SC activity:

In addition to the cytokines mentioned above, SC activity is also mediated by growth factors, which are released by various cell types and the myofiber itself. Each growth factor binds to its specific receptor on the SC and mediates certain cellular processes. The detailed effects of different growth factors have mainly been studied *in vitro* or in rodent models and, unless otherwise stated, the literature reviewed below does not include studies in human volunteers.

2.6.4.1 Hepatocyte growth factor

Hepatocyte growth factor (HGF) binds to its receptor, namely c-met, on the surface of the SC. Exogenous HGF increases the c-met gene expression in SCs (132). Upon binding with c-met, there is a series of reactions which ultimately results in the phosphorylation of MAPK/PI3K pathway (133). HGF regulates the SC by the upregulation of transcription factors responsible for activation and proliferation whilst inhibiting differentiation (132). A negative feedback mechanism results in a decrease in HGF activity (133). In recreationally active volunteers who completed 300 quadriceps eccentric contractions at controlled speed, serum HGF increased early (4 hours after) and muscle hepatocyte inhibitory protein was upregulated 1 day after eccentric exercise and high levels remained for 3 days (134). In summary, HGF is pivotal in the activation and proliferation of SCs whilst inhibiting differentiation to allow for later optimal differentiation-assisted myogenesis, which in turn requires a downregulation of HGF.

2.6.4.2 Insulin like growth factor

Insulin like growth factor (IGF) is known to promote cell growth in almost every cell type in mammalian systems and has multiple iso-forms (135). One of the first observations of IGF-I in skeletal muscle in humans was in 1996 when it was noticed that there is an increase in IGF-I following strenuous exercise (136) yet its different roles were not defined by this study. Besides being present in circulation, IGF-I is released by the extracellular matrix and binds to its receptor IGFR-I, on the SC surface. IGF-I carries out its effects through the Akt/mTOR pathway of protein synthesis, whilst inhibiting protein degradation pathways myostatin and the E3 ligases genes MURF-1 and MAFbx (137, 138) further promoting cell growth.

Following exercise in rodents, IGF binding affinity to its receptor was increased in older animals (139). In human volunteers, IGF-I is found to be upregulated within the cytoplasm of the muscle fibre (140) and to co-localise with its receptor on the SC (141).

Unlike HGF, IGF-I is proposed to have a dual effect on myogenesis by upregulating both proliferation and differentiation, although it is likely that different iso-forms of IGF-I are responsible for either proliferation or differentiation of the SC. Mechano growth factor (MGF) a splice variant of IGF-I promotes SC proliferation and inhibits differentiation (142). The role of IGF-I binding protein 3 was assessed in cultured primary myoblasts. Treatment of IGFBP-3 enhanced myoblast differentiation, even in situations when the cells were treated to inhibit differentiation (143). The role of IGF-I and its splice variants is complex and tightly regulated. It provides a potential therapeutic option for regeneration and treatment for myopathies.

2.6.4.3 Brain derived neurotrophic factor

Brain derived neurotrophic factor (BDNF) levels increase in the brain (mice) and in skeletal muscle (human volunteers) following exercise and plays a role in multiple processes (144, 145). In the context of the SC, BDNF is released by the SC (149) and may act in a paracrine fashion, binding to its receptor, p75NTR on the SC inhibiting differentiation (146). There are no studies that have investigated BDNF and its role on SC activity in humans following an exercise intervention, so its role in response to various types of exercise protocols is not entirely understood.

2.6.4.4 Fibroblastic growth factor

Fibroblastic growth factor (FGF) has multiple isoforms. In skeletal muscle, FGF-2 and 6 localise within the ECM (147) and are proposed to regulate SC activity. In the context of the SC, FGF-2 also known as basic FGF, binds to its receptor on the SC. One of its functions is the inhibition of differentiation and enhancement proliferation (148, 149, 150) via the p38/MAPK/ERK signalling cascade (148). The exact role FGF-6 plays on SC activity is not yet conclusive. It has been proposed that it enhances SC proliferation similar to FGF-2 (151, 152) but it has also been shown that FGF-6 knockout mice have no attenuation of muscle

regeneration suggesting FGF-6 is not crucial for regeneration (153). Results from knockout models must be interpreted with caution, especially in an attempt to understand the role of growth factors on SC activity. A reduction in one growth factor could result in other factors taking over the role of the knocked-out factor. Limited research has been performed on FGFs and SCs in humans following exercise, most likely due to the complexity of the signalling and varying results from cell culture models. Nonetheless, FGF2 may be unresponsive to endurance exercise (154)

2.6.4.5 IL-6

As mentioned above in section 2.6.3, IL- 6 is always associated with inflammation and was initially considered a cytokine as it is released by inflammatory cells to induce a pro-inflammatory state. Now it is known that IL-6 is secreted by many cell types other than immune cells, such as adipose tissue, hepatocytes in the liver and skeletal muscle. In the field of skeletal muscle physiology, IL-6 has been named a myokine as it is released by contracting muscle even in the absence of damage (155, 156, 157). Since the focus of this thesis is high intensity exercise and satellite cell responses, this section will not review all the literature on exercise, skeletal muscle and IL-6; or the literature focused on exercise immunology.

The concept of IL-6 playing a role in SC activity is not a novel concept. One of the first descriptions of the relationship between the two came in 1995 where it was described that there was enhanced myoblast proliferation due to IL-6 (158). In cell culture models, IL-6 has been reported to induce SC proliferation through the JAK2/STAT3/cyclin D1 signalling pathway, which is regulated by a negative feedback mechanism with the upregulation of SOCS3 (159). IL-6 binds to its receptor (IL-6R) and gp130 to form a complex, phosphorylating the intracellular domain of gp130 and subsequent phosphorylation of JAK and STAT, where pSTAT dimerises and translocates into the cell nucleus targeting proliferating genes (160). Suggesting that IL-6 performs its role in a paracrine and autocrine manner.

In vivo models have demonstrated that following eccentric exercise-induced muscle damage there is an increase in IL-6 and its receptors, co-localising with SCs after exercise (161) and an increase and co-localization of, pSTAT3 and SCs 1, 3 and 24 hours after eccentric exercise (162). If the proper regulation of the JAK/STAT pathway is not achieved, it may lead to negative downstream effects. Genetic deficiencies resulting in a lack of IL-6 in mice, are associated with a decreased SC function and hypertrophy (163). In contrast, hyper activation of the pathway activates the ubiquitin protease pathway and subsequent muscle wasting. Hyper activation of the pathway has been noted in diseases such as dystrophy (164) and cachexia (165). Further review of the IL-6 literature is beyond the focus of this thesis.

Growth factors as potential therapeutic options:

A study done on rabbit *superior rectus* muscle investigated the effect of HGF injections alone and in synergy with IGF-I injections on skeletal muscle growth and force production. The results showed that HGF alone promoted no increase in fibre CSA nor increased force production, but it showed an increased number of SCs. An HGF injection followed by an injection of IGF-I one week later produced an increased fibre CSA, force production and increased SC number to a much larger extent than IGF-I in isolation. (166). These results can be explained using the basic knowledge of each of the growth factors, as outlined above. While HGF activates quiescent SCs it inhibits differentiation and ultimately the SC won't fuse with the myofiber. IGF-I on the other hand is known to play a role in muscle hypertrophy by contributing to SC induced hypertrophy but also influencing protein synthesis pathways. The synergistic treatment allows HGF to activate SCs and increase the SC pool size, and IGF-I to further move the SCs down the myogenic process while increasing protein synthesis and growth pathways. Understanding the mechanisms of these growth factors that affect SC activity may have potential as treatment options to enhance skeletal muscle regeneration in an athletic and therapeutic context. It must be considered that growth factor injection alone may not induce the desired effect as the SC may not express the membrane receptor so that binding may occur (167). Also, in the athletic context, banned substances should be replaced by effective training protocols which would naturally induce a growth factor response and subsequent adaptation within the muscle.

2.6.5 Satellite cells and response to a single bout of exercise

Although the main focus of this thesis is the SC response to different HIT regimens, it is important to understand the SC response to a single bout of exercise as the muscle has not yet adapted to the exercise. When the main muscle groups used are unaccustomed to the exercise stimulus they are vulnerable to muscle damage e.g. post a resistance training bout or eccentric exercise. Following a single bout of resistance training, IGF-I co-localises with SCs, activating them to propagate along the myogenic pathway (141). As already mentioned, increases in the SC pool size have been reported at 12, 48 and 72 hours after exercise, as activated SCs rapidly proliferate in response to the exercise (168). mRNA of Myf5, MyoD and Myogenin are upregulated as soon as 3 hours after an acute bout of resistance training (169) and Snijders et al. have reported myoD positive SCs at 12, 48 and 72 hours. In muscle fibres themselves, there is an increase in signalling proteins of growth pathways, such as mTOR. The SC response may be involved in healing the muscle readying it for a subsequent bout of exercise. In contrast, in aged muscle the SC response to acute exercise is not as potent as younger individuals. This is potentially due to the fact that the resting SC pool size is smaller in older individuals, resulting in a less potent response to exercise, as pro-growth responses, such as IGF-1 gene expression, are most likely dysregulated in the aged muscle (170). Different types of exercise may elicit different responses. A single bout of endurance exercise at 40% VO_2max increased myogenin content immediately after exercise in the myonuclei but not in the SC (171). One wouldn't expect to see SC differentiation so soon after exercise, yet this reported result is interesting for two reasons: 1, the myonuclei responded immediately after exercise and 2, Myogenin expression is usually considered to be linked to SCs and not myonuclei. For reviews discussing the effect of a single bout of exercise on SCs, see (172, 173, 174). The mechanisms may include mechanical signals, autocrine or paracrine factors, or all of these, which stimulate rapid proliferation of the SCs to promote muscle regeneration, although many of the studies have not assessed muscle damage directly.

2.6.6 Satellite cells and the response to eccentric vs concentric contractions

As shown above it has been established that there is a SC response to exercise, but less is known about SC activity as a result of exercise modality. Contractions types result in different levels of growth factors being released. There is a larger upregulation of IGF-1, which is known to influence SC proliferation and differentiation, after a concentric resistance exercise bout compared to an eccentric resistance bout (175). Eccentric exercise is known to be more damaging to the muscle compared to concentric exercise, and there is an increase in SC numbers following acute eccentric exercise to repair the damage (176). Exercise training seems to display a different SC response. Concentric knee extension resistance training resulted in a larger increase in the SC pool of both type I-specific and type II-specific SCs with 12 weeks of training when compared to eccentric knee extension resistance training (177). This suggests that after an acute eccentric bout there is damage, SC-induced muscle repair with fusion of SCs to the adult fibres and return to quiescence of sufficient numbers to maintain SC pool size. However, due to the fusion events, the SC pool size does not exhibit a lasting increase in response to an acute bout and this is evident depending on how long after the bout the muscle biopsy was taken. On the other hand, exercise training may induce mechanisms that result in an increased SC pool size over time.

2.6.7 Satellite cells, exercise training and hypertrophy

Skeletal muscle is unique in the sense that it has a large number of nuclei per cell, or muscle fibre. Myonuclei are distributed along the muscle fibre and the notion is that each nucleus has control over a certain area of cytoplasm in which protein synthesis occurs. This area of cytoplasm is known as the myonuclear domain (MND) (178, 179). In essence, the theory suggests that the MND size is consistent throughout the muscle fibre in resting muscles but with the introduction of external stimuli that affect muscle fibre size such as overload leading to hypertrophy or conversely atrophy from disuse and disease, the MND size is temporarily altered and the number of myonuclei must then adjust so that the MND size is returned (180). However, the size of the MND varies between fibre types, with a larger MND in type II fibres compared with type I fibres. The MND size may increase with muscle hypertrophy

without initial adjustment but has seen to have an upper limit where no further increase in MND size occurs. Addition of nuclei is as a result of activation and proliferation of SCs and their fusion into the muscle fibre. This increases the number of myonuclei thus impacting the MND size during the compensatory time (181). Research into MND size alteration has provided contradicting results and as reviewed by Herman-Montemayor *et al* (2015), some studies report that strength training results in an increase in MND size, hence little or no fusion of SCs, while others report hypertrophy with little effect on the MND size, hence adjustment of total myonuclear number (181). The size alterations of the MND in multiple states questions the efficacy of the paradigm (182). Understanding SC responses to the intervention at the mechanistic level instead of only assessing MND size, sheds more light on the topic.

It has been thought of for quite some time that hypertrophy requires some SC activation and progression down the myogenic pathway until subsequent fusion into the myofiber as the addition of a myonucleus. Petrella et al., reported that in response to a 16-week resistance training programme, the SC pool size increased by 117% post training, and that there was an increase in myonuclear content of 29% in the responder group, while the non-responders showed no increase to both SC pool increase and myonuclear addition (183). Individuals who had a higher baseline SC pool size showed an increased ability to expand the existing SC pool size and incorporate new myonuclei into the myofiber. This phenomenon is known as hypertrophic or myogenic potential (183) and can be considered as an accelerated myogenic response following exercise. A SC pool expansion has also been noted in elderly men who underwent a resistance training programme for 16 weeks (184). Variations of resistance training, such as training with addition of blood flow resistance, increased the SC pool size four-fold, as well as the myonuclear content and fibre CSA (185). See able 2.3 for a summary of the effect of training on SC content.

Studies that show an increase in SC pool size and myonuclear number per fibre conclude that SCs are activated and proliferate due to training, and fuse into the myofiber. The question is whether or not this is causing an increase in CSA, i.e. can the CSA increase without the addition of myonuclei? Is a result of an initial increase in CSA and thus an increase in the myonuclear domain placing an an extra cytoplasmic load on the existing myonucleus leading

to myoblast fusion into the myofiber? In human studies with the inherent ethical limitation on the number of biopsies that can be taken interpretation is difficult. If a typical 16-week resistance training protocol increases CSA as would be expected, with no changes to the myonuclear domain, this implies that the SC pool size and myonuclear number increased over the training protocol, but unless a transient increase in CSA is seen before increase in myonuclei this does not suggest that an increased myonuclear domain is necessary to drive the fusion of myonuclei into the myofiber (186). Researchers using animal models of hypertrophy suggest that there may be no need for SC to ultimately undergo fusion into the myofiber to become a myonucleus, in order to contribute towards hypertrophy. It has been reported in rat soleus that unloading, using hind limb suspension and reloading thereafter after resulted in a decrease and increase in CSA respectively but no change in myonuclear number per fibre in either situation (187). A mouse model (Pax7-DTA) that is engineered to have approximately 90% of its SC pool depleted has shed some light on this topic. When subjected to mechanical strain, there was an increase in muscle fibre CSA and force production to a similar extent to that of normal mouse muscle, but without the increase in myonuclear number post mechanical load (188). Whilst this questions the significance of the SCs role in hypertrophy, due to the dynamic nature of skeletal muscle, other growth mechanisms were probably called upon to potentially take over the role of the SC. Trying to translate this mouse model to human muscle adaptation in a physiological context (i.e. without genetic engineering) may be challenging.

It is clear that SCs are of the utmost importance to muscle fibre regeneration due to injury and formation of new myofibres during hyperplasia but perhaps hypertrophy is not necessarily mediated by SCs but instead by the myonuclei upregulating growth and protein synthesis pathways. However, as the SC responds to mechanical strain and is subject to communication by growth factors, it is not surprising that there is altered SC activity due to exercise regimens that have these effects. See table 2.3 for a summary of the SC response to exercise training, with interventions that include traditional resistance training, aerobic cycle training with the duration of training spanning from 6 – 16 weeks. What can be noted is that the increase in the SC pool in response to training ranges from 56 to 533%, suggesting a very specific SC response to training types, durations and intensities.

Table 2.3. Satellite cell induced muscle adaptation

Model	Muscle Biopsy	Intervention	Intervention details	SC number/ Fibre	Reference
Male, young, unaccustomed to resistance training	*Vastus Lateralis*	Resistance training	Leg RT 3 months of RT. 3 x a week. 6-12 RM	35% ↑	(118)
Female, young, sedentary	*Vastus Lateralis*	Aerobic interval training	Cycle Ergometer 6 weeks 3 x a week 10x60sec 90% HR_{max}	Type I: 19% ↑ Type II: 31% ↑ Hybrid: 533% ↑	(113)
Male and female sedentary adults,	*Vastus Lateralis*	Aerobic training	Cycle Ergometer 12 weeks 3 x a week 70% $HR_{reserve}$	56% ↑	(189)
Male, young, unaccustomed to resistance training	*Vastus Lateralis*	Resistance training	Leg RT 16 weeks 3 leg exercises 4x8 repetitions 80% 1RM	Type I: 40% ↑ Type II: 66% ↑	(114)
Male, young, unaccustomed to resistance training	*Vastus Lateralis*	Blood flow Occlusion resistance training	Knee Extensions 8 weeks 4 x 20% 1RM to failure	272% ↑	(185)

RT Resistance training. AT Aerobic training. RM Maximal repetition. HR heart rate

2.6.8 Satellite cell activity in response to running

Less SC research has been done in exercise training models other than resistance training, most likely due to the abundance of unanswered questions and also since resistance training is known to give a very potent SC response. As stated before, downhill running is known to be eccentrically biased exercise for the *quadriceps* muscles and therefore causes exercise induced muscle damage when unaccustomed. There is an increased SC number 24 hours after a bout of downhill running, most likely to repair the muscle damage (120) and an increased SC pool 1 week after of recovery from a single bout (190). No published work done on the role of satellite cells in uphill running in humans, and only one paper done on a rodent treadmill model (191). A few rodent models have been used to induce SC activity due to running. Following voluntary wheel running, which is considered to be relatively less stressful on the muscle as opposed to gradient running, increased Wnt signalling has been reported, upregulating mRNA transcription of Myf5 and MyoD (192) suggesting SC activation and proliferation due to running. The effect of downhill running on SC dynamics was done by Darr et al. who showed that there was exercise-induced muscle damage and an increase in active SCs 24 hours post-exercise (193). More studies need to be conducted in human volunteers to address the SC response to running, in terms of the intensity, duration and gradient and their effects on both the SC pool and muscle fibres themselves to gain a better understanding of how different exercise modes and the variety thereof may result in varying muscular adaptation.

2.6.9 Satellite cell activity and intensity of exercise

As mentioned above, the type of exercise, duration and intensity are a few variables to consider when designing an exercise protocol, as these may well add variability to the results, which would be assessed best when trying to compare between exercise protocols in the same study. There is a limited body of literature providing evidence in terms of SC dynamics and intensity as a regulator of SC dynamics as opposed to duration/volume. High intensity exercise increased SC numbers 4 and 8 days after exercise, with no signs of muscle damage assessed by visualisation of desmin and dystrophin in biopsy samples. The authors concluded that the SC activation was due to unaccustomed intensity of exercise and not muscle damage (194). In contrast, a study by Joanisse et al. (2015) concluded that there is no change to the

SC pool following a low load, high intensity sprint training protocol relative to a moderate intensity continuous training protocol and that both intensities of exercise activate SCs (195). It was unclear from this study as to how the two exercising groups were matched for comparison. The intensity, duration and distance differed. Perhaps if the protocol was matched at the same distance covered by the two protocols the SC response may have been different. There are no human studies which address intensity vs duration as the main stimulus for SC activity. A rodent model used to compare training intensities showed consisted of 4 training groups. Group 1: low intensity, low duration, group 2: low intensity high duration, group 3 high intensity, low duration and group 4: high intensity high duration. The exercise was performed on a rodent treadmill and the running was continuous with only the duration or intensity differing. Following the training protocol there were no differences in CSA or myonuclei number between any of the groups. When assessing the SC response, both the high intensity groups had a larger SC pool size compared to the low intensity groups, regardless of duration. These results suggest that intensity may be a more potent factor than duration to increase the SC pool (196).

2.6.10 Satellite cells and supplements, steroids and NSAIDS

In addition to the responsiveness to growth factors mentioned above, SC are subject to communication by immune factors in the environment and other nearby SCs and it would not be surprising if there is altered SC activity due to supplementation, medication or anabolic drug use. Non-steroidal anti-inflammatory drugs (NSAIDS) are consumed by athletes and sedentary individuals in an attempt to alleviate muscle pain. NSAIDS have been hypothesized to alter SC activity by modifying growth factor release and by altering growth pathways that would need prostaglandin (197). When assessing SC activity in a human model, caution must be taken in order to ensure that the study participants are not on any drugs which could alter the results of the study.

2.7 Capillaries

2.7.1 Structure and Function of capillaries:

Blood supply to the skeletal muscle is characterized by dense vasculature with arterioles feeding from the arteries and the capillaries running between the fibres supplying the adjacent muscle fibres with oxygen and nutrients, as well as taking metabolic waste nutrients away from the muscle (198). In addition to this pipe-like format, capillaries form a network of twisting narrow vessels connecting capillaries. These twists enable them to cover a larger surface area compared to if they were arranged just in a straight line this phenomenon is known as capillary tortuosity (199). Due to the different metabolic requirements of the isoforms of myofibres, capillarisation may alter throughout the muscle with relatively more capillaries present adjacent to the more oxidative type I fibres compared to type II fibres (200). A study assessing rat muscle observed that the highly active *soleus* consists of a denser capillary network compared to the less active *plantar flexor*. Both muscles display tortuosity, but higher tortuosity is observed in the *soleus* (201). Capillaries are the smallest units of the vasculature and there are so many that they have been estimated to be 2-3% of skeletal muscle mass (202).

Due to structural identification playing such a crucial role in understanding the capillary network, the most common technique used to identify capillaries is through immunohistochemistry staining for visualisation by light microscopy or immunofluorescence microscopy. The most common marker used for capillary identification is the endothelial cell marker CD31 (203). Other markers used to identify capillaries in skeletal muscle include collagen type IV, lectin, von Willebrand factor, fibronectin, and wheat germ agglutinin (WGA) (204). Histochemical staining methods that have been used to identify capillaries are the Toluidine blue stain (205) which stains the muscle fibres and outlines the capillaries, or amylase-periodic acid Schiff staining (204). For confirmation of capillary identification, combinations of the above identification methods have been used.

Measuring the capillary network in skeletal muscle in a quantitative fashion is of vital importance to fully understand the change in network, if any. The simplest measurement method is the number of capillary contacts with a muscle fibre, known as the capillary to fibre ratio (C:F) which is usually determined on cross-sections. An untrained healthy male will typically have a C:F value between 1,5 and 2 and this value may increase with endurance training (206). The C:F is usually coupled with the capillary density measurement, which is the number of capillaries in an area of muscle, expressed as number per mm^2. An untrained healthy male will have a capillary density between 350 and 400 x mm^2. In the case of training this value may increase, but because it depends on the CSA of the muscle fibre, there may be an increase in capillarisation but also an increase in muscle fibre size, therefore the capillary density will remain unaltered. In cases of detraining, there may be a decrease in capillarisation but also a decrease in muscle fibre size, again the capillary density will remain the same. There may not necessarily be an increase in C:F or CD with training, but that may not fully explain the extent of capillary network behaviour. A capillary tortuosity index has been described by Huntsman et al., as the length of capillaries in contact with each fibre (207). This measurement indicates whether capillary growth is induced with training, or whether the already existing network may become more tortuous to improve nutrient and oxygen diffusion before capillary growth is induced. A recommendation for assessing muscle capillarisation is by analysing multiple serial sections to quantify tortuosity. A comprehensive measurement of the capillary network in skeletal muscle is the capillary-to-fibre-perimeter exchange index (CFPE) developed by Hepple in 1997. The CFPE is a quotient of the number of capillary to fibre contacts and the muscle fibre perimeter (208). To try to understand the capillary network, a combination of the above measurement methods should be used to give more information before conclusions can be made.

As mentioned above, identification and measurement of the capillary network is primarily done in cross sections. This is done due to the difficulty of identifying capillaries in longitudinal orientation since they do not follow the proximal to distal orientation exactly. Perhaps if the capillary network was measured in both orientations it could elucidate a more precise story. Attempts to address this issue have been made by the construction of 3 dimensional (3D) imaging of the capillary network and comparing this to 2D identification methods. Serial sections of mouse *gastrocnemius* were imaged, analysed and overlaid on software generating the 3D image. It was noted that there was a degree of error in the 2D

identification compared to the 3D image (209). This method, however, is time and labour intensive and expensive.

2.7.2 Exercise induced angiogenesis

The capillary network in skeletal muscle is subject to change according to stimuli. Angiogenesis is the formation of capillaries from existing blood vessels, as opposed to vasculogenesis which is the *de novo* formation of the larger blood vessels (210). The capillary network under resting conditions is stable and quiescent, until activated by an external stress such as exercise training which induces angiogenesis, expanding the capillary network.

Angiogenesis may result from various stimuli, such as mechanical stress of the muscle or strain on the capillaries themselves (211), hypoxia (212), insufficient delivery of nutrients for metabolism, or insufficient removal of metabolites resulting from exercise (213). It is a complex and highly regulated process which may occur by one of two mechanisms. Sprouting angiogenesis involves proteolytic degradation of the basement membrane of the capillary and proliferation of the endothelial cells sprouting to form new capillaries (214). Non-sprouting angiogenesis on the other hand, does not involve degradation of the basement membrane and subsequent proliferation of endothelial cells. Rather it occurs when a protrusion from the endothelial wall splits the capillary into two channels and eventually splits into two capillaries (215). Angiogenesis is regulated by factors promoting and inhibiting angiogenesis. The levels of these factors, which will be explained below, may differ in skeletal muscle at rest, during and after exercise.

2.7.2.1 Angiogenic signals and response to exercise

Vascular endothelial growth factor (VEGF) binds to its receptor (VEGFR) inducing a cascade of cellular signalling with downstream effects on endothelial cell gene transcription for cell survival and proliferation (216). VEGF may be located in skeletal muscle or endothelial cells or pericytes, or all of these cell types. VEGF induced proliferation of endothelial cells alone will not result in angiogenesis as there is no change to the endothelial cell basement membrane. Matrix metalloproteinases (MMPs) are responsible for the

proteolysis of the basement membranes around endothelial cells, allowing sprouting angiogenesis to take place (217). A feedback mechanism exists whereby tissue inhibitor of MMP (TIMMP) acts to inhibit the angiogenic effects of MMP. Angiopoetins (ANGPT) 1 and 2 compete for binding to the receptor Tie2. ANGPT1 Tie2 binding has downstream effects of a stronger bond between endothelial cells, which represents an anti-angiogenic state (202), whereas ANGPT2 Tie2 binding inhibits the downstream effects of endothelial cell interactions and causes capillary de-stabilisation. In combination with VEGF activity this will allow angiogenesis to take place.

After an acute bout of exercise there is an increase in VEGF mRNA as soon as 45 minutes after cessation of exercise (218) and VEGF protein levels have been reported to be increased already at 120 minutes after exercise (217). MMP 2, 9 and 14 are also upregulated after an acute bout of exercise (217). Acute exercise does not increase ANGPT2 levels (219). Muscle interstitial fluid taken immediately following an intense exercise bout was insufficient to induce proliferation of endothelial cells *in vitro* (220). It is likely that increases in VEGF-A return to baseline after exercise or that an acute bout of exercise is an insufficient stimulus for all the necessary pathways to be active resulting in angiogenesis.

With exercise training there is an increase in basal levels of VEGF-A and its receptor (221) as well as ANGPT2 and Tie2 protein levels (219). The repetitive exercise bouts are sufficient to allow for both endothelial cell proliferation and proteolysis of the basement membrane, resulting in angiogenesis and an increased muscle perfusion. See Table 2.4 for a representative summary of studies on muscle capillary adaptation as a result of training in human volunteers, which indicates that the duration of interventions was typically 8 weeks but extended to 12, 16 and 26 weeks for a few studies. Also aerobic interval training appeared to be most effective for increasing C:F ratio in cyclists. Lastly, with longer slower aerobic training, the adaptation (expressed as % change) seemed to correspond when comparing C:F and CD, which was not the case with the interventions that may have been more likely to increase CSA.

Table 2.4: Muscle capillary changes with exercise training

Subject Description	Training type	Exercise intervention	C:F (%↑)	CD (%↑)	CFPE (%↑)	Reference
Sedentary males	Aerobic training: Running	6 months 4 sessions per week 30min 75% VO_2max	25	20	N/A	(206)
Sedentary males	Aerobic training: Running	8 weeks 3 sessions per week Various session types: HIIT, Fartlek, Long slow distance	14	N/A	N/A	(222)
Sedentary males	Aerobic training: Cycling	8 weeks 4 sessions per week 1 hour 65% VO_2max	25	20	21	(223)
Sedentary males	Aerobic interval training: Cycling	8 weeks 5 sessions per week 30 min 60-95% HR_{max}	39	13	N/A	(226)
Recreational male runners	Sprint interval training: Running	8 weeks 2 sessions per week 4x 5 1minute sprint intervals at 30,60 and 100% speed	0	0	N/A	(224)
Sedentary males	Resistance training	16 weeks 4 sessions per week Upper and lower body exercises 6 sets 65-85% RM	16	7	7	(114)
Healthy old males	Resistance training	12 weeks 3 sessions per week Lower body exercises 4 sets 75-80% RM	20	11	N/A	(225)

HIIT High-intensity interval training HR Heart rate RM Maximal repetition C:F Capillary to fibre ratio CD Capillary density CFPE Capillary-to-fibre-perimeter exchange index

2.8 Satellite cell association with Capillaries

In response to muscle damage regeneration is accompanied by the SC response, as previously discussed, but also an increase in blood flow may be stimulated by factors released in response to damage. SCs localize in close proximity with capillaries under resting conditions (227) in exercise stimulated muscles and regenerating muscle (203), Quiescent SCs may localise near a capillary for general cell maintenance and an accelerated response to growth factors in circulation. SC activation due to exercise appears to be specific to the position of the SC, with the activated SCs found to be nearer to capillaries compared to quiescent SCs (203) (114). Individuals with enhanced baseline muscle capillary content had greater SC pool proliferation and differentiation following a muscle damaging bout of exercise compared to individuals with lower muscle capillary content (228). This morphological relationship could suggest that there is a relationship between angiogenesis and myogenesis (229).

Angiogenesis and myogenesis occur in parallel with each other during muscle regeneration, which has been explained by *in vitro* (230) and *in vivo* models (231). To investigate whether angiogenesis and myogenesis are coupled or merely occur temporally in parallel, Borselli et al., (2010) introduced both VEGF and IGF-1 intravenously to ischemic hind-limb mouse muscle. The combination of both VEGF and IGF-1 resulted in greater regeneration, revascularisation and a greater force output compared to individual treatment, suggesting an interplay between the two processes. (232).

Endothelial cells have been proposed to increase proliferation of SC in co-culture models *in vitro*, which could be due to the secretion of VEGF by the endothelial cells. This then has a mitogenic and anti-apoptotic effect on the myoblasts (233). The effect of the angiogenic factor, Ang-1 (the protein product of the ANGPT-1 gene), on SCs was measured both *in vivo* and *in vitro* by Mofarrahi et al. (2015). Ang-1 levels were increased in SCs 2 weeks after cardiotoxin induced muscle damage in rat muscle (234). The same study provided evidence that recombinant Ang-1 added to primary human myoblasts enhanced cell survival (measured with a cytotoxicity assay), migration (measured using a scratch wound assay), proliferation (measured using a BrdU immunofluorescence absorption assay) and differentiation (measured by qPCR analysis of myoD and myogenin RNA) (234). This provided insight to

how angiogenesis may influence myogenesis. On the other hand, Rhoads et al. (2009) aimed to investigate the potential role of the SC on angiogenesis. Sprague-Dawley rat SCs were isolated and cultured. SC-conditioned media was added to microvascular fragments in a separate culture and the resulting in enhanced angiogenesis, measured by the number and length of smooth vascular sprouts present on the microvascular fragments (235). In addition to the cell types mentioned earlier as sources of VEGF, SCs are known to secrete VEGF. VEGF receptors were added to the SC conditioned media before being applied to the microvascular fragments, blocking the effect the VEGF from the conditioned media might have. This resulted in a blunted angiogenic response. These results suggest that SCs influence angiogenesis via VEGF. Furthermore, other cells of the vasculature such as smooth muscle, are involved with the re-entry into quiescence of a proliferating SC (233). To explore this further, *in vivo* models that characterise both myogenesis and angiogenesis need to be designed, especially to look at the cross talk between the processes.

2.9 Summary

Exercise training results in muscular adaptation, which leads to improved performance. The SCs and the vasculature may be at the core of this muscular adaptation. Traditionally training studies which assess the SCs embrace a resistance exercise or aerobic cycle training protocol, with fewer studies using a running protocol. Studies that assess the impact of intensity on muscular adaptation commonly involve cycling training. There is limited research on the role of high intensity interval run training on the SC response. Additionally, concentric and eccentric contraction forms, which can be achieved by running either uphill or downhill respectively, may cause different muscular adaptation. The differing adaptation may, however, both act through the SC activity. In the context of HIIT, literature includes studies which assess performance indicators as a product of HIIT but not SC-related muscular adaptation. This study is the first study which compares the muscular adaptation due to either an uphill or downhill running HIIT protocol and also assesses SC dynamics at different time points.

Chapter 3: Hypothesis, aims and objectives

Hypothesis:

The different modes of high-intensity interval training will result in improved muscle performance and may result differing skeletal muscle damage and satellite cell activity. Downhill running will induce more extensive muscle damage and a more potent SC response than uphill running, whilst uphill training will induce angiogenesis to a larger extent when compared to downhill training.

Aims:

To determine selected aspects of muscle adaptation, such as aerobic capacity, muscle strength, running performance, muscle fibre morphology, satellite cell activity and vascularization following 4 weeks of either concentric or eccentrically-biased high intensity training in well-trained, but not elite subjects.

Objectives:

Muscle adaptation was induced with the implementation of a 4 week uphill or downhill HIIT intervention. Muscle biopsies and blood draws were collected at selected time points to assess the muscular adaptation. The muscular was visualised to assess the extent of muscle damage, muscle fibre morphology, as well as the SC and capillary response to training, using fluorescent microscopy. Biochemical analysis for levels of selected MRFs was used to assess the SC activity further. To assess aerobic capacity, VO_{2max} was tested using a flat incremental exhaustive treadmill exercise test. Quadriceps muscle strength testing was achieved with the use an isometric chair. Running performance was assessed by a 5km road time trial.

Chapter 4: Methods and materials

4.1 Ethical Consideration

The research project was ethically cleared by Stellenbosch Health Research Ethics Committee (#1781, Appendix A) and was performed within the guidelines set by the South African Medical Research Council, which are based on the declaration of Helsinki.

4.2 Study Design

4.2.2 Subjects

Sixteen fit but not elite male subjects were recruited from the Stellenbosch area by means of a flyer (appendix B). In order to be recruited for the study strict inclusion and exclusion criteria were set.

Inclusion criteria:

1. Age between 18 and 30 years
2. Must be able to run 5 km in a time between 20 and 25 minutes
3. Unaccustomed to eccentric exercise
4. Able to understand the circumstances of the study, thereafter signing informed consent

Exclusion criteria:

1. Participation in professional sport
2. Participation in intensive hiking or hill walking
3. Use of anti-inflammatory medication within 3 months preceding the study
4. Use of chronic medication
5. Illness within three weeks of the study
6. Smoking

Subjects were randomly assigned to an uphill-running group (n=5) and a downhill-running group (n=6). To achieve randomisation and even distribution between groups. Subjects were given a subject ID number upon entrance into the study. The subjects then completed the baseline 5km time trial and times were ordered in descending Subject IDs were evenly distributed into either the DHG or the UHG according to the descending order of the 5km baseline time trial. Subjects were given an exercise logbook, where they logged training that they were partaking in for the 4 weeks prior to the study and for the duration of the study all

training excluding the laboratory sessions to ensure that the HIIT intervention was the stimulus for adaptation and not any additional training.

4.2.3 Study outline

The study protocol involved three phases. Phase 1; baseline performance testing, baseline muscle biopsy and blood sampling. Phase 2; 10 high intensity interval training (HIIT) sessions were completed over a four-week period. A blood sample was collected at the beginning of every week and a muscle biopsy was taken at the end of the phase. Phase 3; post-training performance testing. The study design is summarized in figure 4.1.

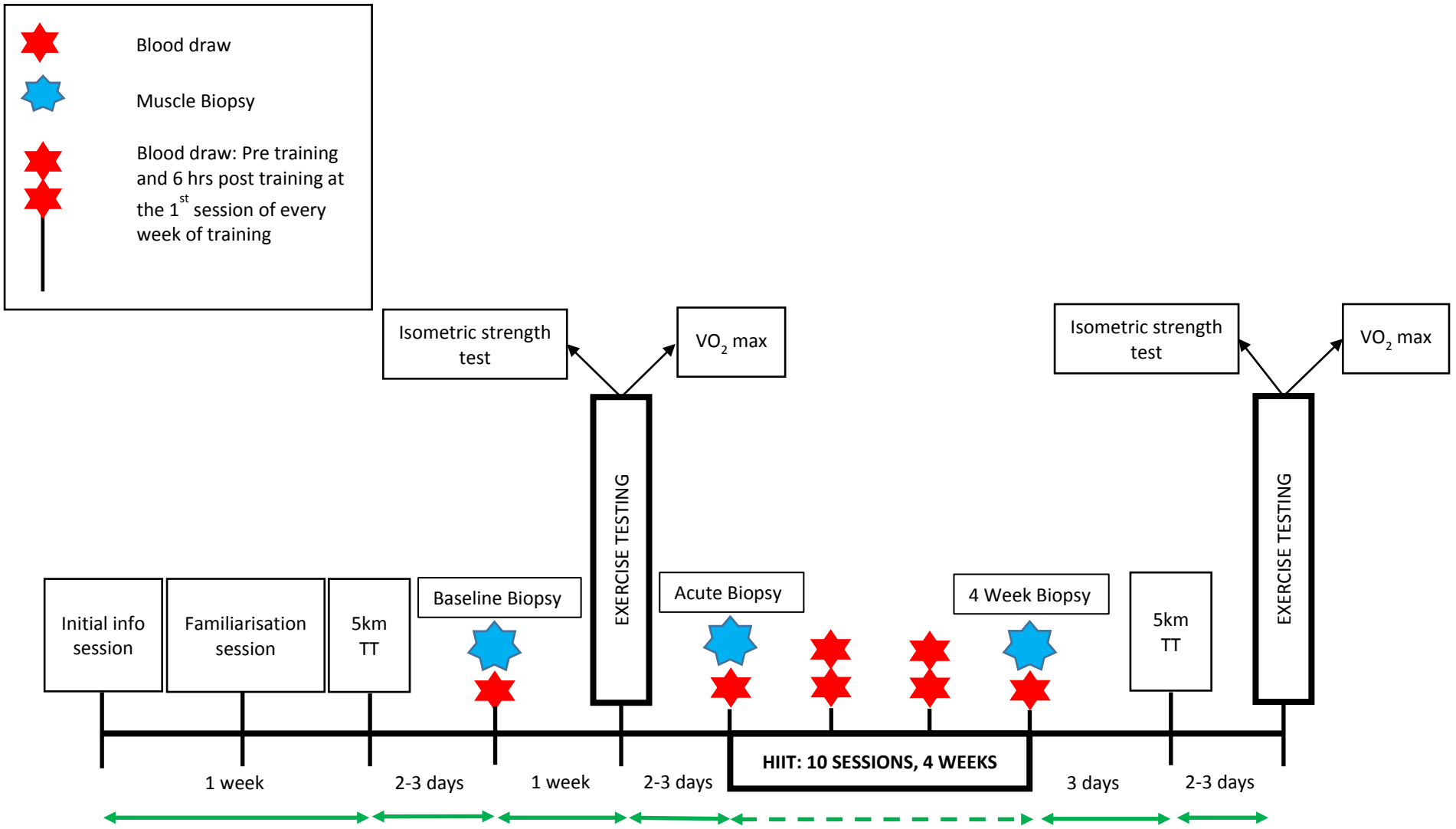


Figure 4.1 Scheme of the study time line

4.2.4 Initial information session and Familiarisation of lab equipment

Subjects were invited to the exercise laboratory to take part in a familiarisation session. This session consisted of a treadmill warm up at the desired pace and duration of the subject. The subjects were familiarised with the testing protocols of both the maximal isometric strength test and treadmill VO_{2max} test. The subjects were able to decide which size face mask and headgear was most comfortable for the VO_2 incremental familiarisation test, which was not performed until exhaustion, but terminated when the HR of the subject reached 160 bpm.

4.3 Performance testing

Subjects underwent performance testing 1-2 weeks before the baseline biopsy. The familiarisation session (used to get used to the apparatus and testing protocols, as described above) narrowed down the learned effect from doing an exercise test, so that it can be said with more certainty that post training performance test increase (if any) was due to the training intervention and not the learned effect of the exercise test.

4.3.1 5 km Time trial (TT)

A 5 km road route was mapped out in the Jonkershoek road area with the use of a Global positioning system (GPS). In order to ensure the gradient profile didn't favour the uphill or downhill training group, the route stretched for 2.5 km where there was the turnaround point, and the runners would turn and run back along the same route to finish at the start line. The gradient of the route is illustrated in the image below. Each subject completed their time trial individually in order to eliminate the chance of a fast or a slow time trial caused by running with other runners.

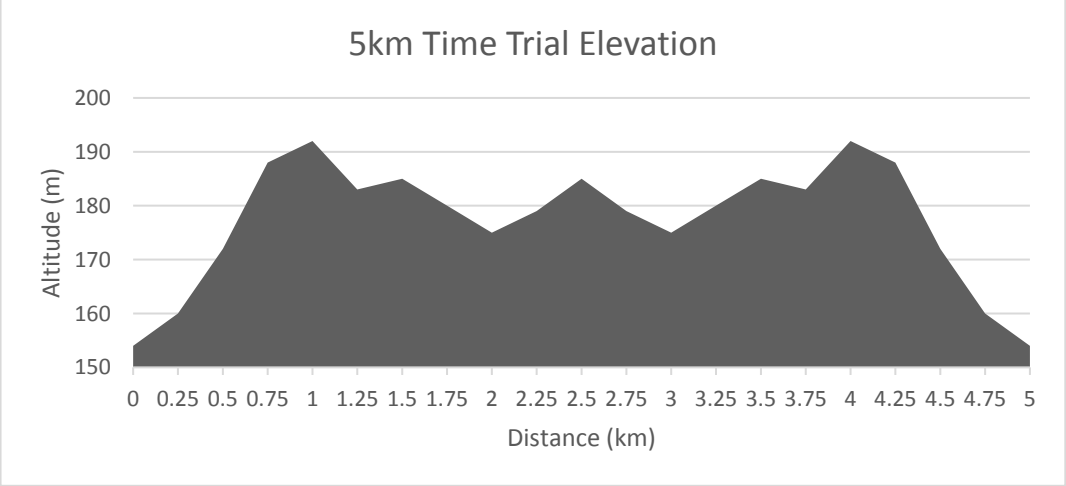


Figure 4.2 Graph illustrating the change in altitude above sea level over the 5km time trial course

4.3.2 Isokinetic strength test

Subjects' muscle strength was tested using an isokinetic chair (Department of engineering, Stellenbosch University, South Africa). The subject was seated in the chair and modifications were made to the chair seat and back rest and force transducer cushioned crossbar positioning for comfort. These parameters were entered into the software which recorded the force output to normalize each individual. The subjects performed a leg extension to a fixed crossbar, which is connected to a force transducer with analogue to digital conversion. The force reader relays the data to the software which generates the data as force output. The test consisted of 3 maximal isometric contractions for 5 seconds each. The peak force output for each attempt was then averaged, and the average force expressed in Newtons (N).

4.3.3 Incremental exercise test until exhaustion

Subjects completed a flat (0 % gradient) incremental test. Subjects wore light running apparel and a heart rate monitor (Polar, s710, Sweden). Warm up was at least 8 minutes on the treadmill at a running speed of their choice. The test started at a standard speed of 10 km/h and increased by 0.5 km/h every 30 seconds, as shown in the table below. The test was completed when the subject was unable to complete a final speed. Termination of the test occurred when a subject reached 20 on the Borg RPE scale, or if they opted out. A positive test result was achieved if the following criteria were all met:

1) A plateau in oxygen consumption (VO_2)
2) A maximal heart rate within 10 beats of their calculated maximum heart rate (220bpm – age)
3) A respiratory exchange ratio greater than 1.10

These parameters were monitored by the investigator and during the test all efforts were made to keep encouraging the subjects to achieve this. There was not a situation where a subject did not meet the positive test criteria.

Table illustrating the protocol for the incremental exercise test until exhaustion

Workload	Time	0% Gradient
1st	30 s	10.0 km/h
2nd	30 s	10.5 km/h
3rd	30 s	11.0 km/h
4th	30 s	11.5 km/h
5th	30 s	12.0 km/h
and more	30 s	+0.5km/h
nth	X s	Final km/h

4.3.4 Breath by breath analysis

An automated gas analyser (OxyCon Pro, Jaeger, Germany) was used to collect gas exchange data. The machine was calibrated for ambient temperature and humidity, volume of air, and gas using 5 % CO_2 (balanced with nitrogen) and room air (0.03 % CO_2 and 21 % O_2).

4.4 Training Intervention

4.4.1 Training diaries

Subjects were given training logbooks 3-4 weeks prior to the start of the protocol, to log all external training from the HIIT intervention. Subjects were told to try to maintain the same training status as before they started the intervention during the weeks of HIIT, in order to ensure adaptation was from the added intervention and not from any added or reduced external training. Subjects were encouraged to be honest in their logbooks.

4.4.2 High Intensity Interval Training

The HIIT sessions were held in the Exercise Lab at the Department of Physiological Sciences Stellenbosch University. The lab's temperature and humidity were set to 24 degrees C and 29% respectively by air conditioning and air exchange with outdoor air. The sessions were completed on an electronically braked treadmill (Runrace, Technogym, Italy). Both groups completed 10 HIIT sessions in a space of 4 weeks, whilst trying to spread the sessions out as evenly as possible to allow for sufficient recovery. Subjects wore a heart rate monitor (Suunto Ambit 3, Switzerland) during HIIT training, to enable comparison of the heart rate responses between groups.

4.4.3 Uphill training

Uphill training was performed at a gradient of 5 % and 80 % of the subject's peak treadmill speed attained during the incremental exercise test. (Peak treadmill speed was not adjusted to the fraction of the final 30 seconds that were completed). Intervals lasted between 2 minutes 30 seconds and 3 minutes with a 1 minute rest. Subjects were encouraged to complete as many intervals as possible or at least 6 intervals. If the subjects comfortably completed 6 intervals lasting 3 minutes each interval, the speed was increased by 0.5 km/h in the following session to challenge the subject.

4.4.4 Downhill training

Downhill training was performed at a gradient of -10 % and 90 % of the subject's peak treadmill speed attained during the incremental exercise test. Intervals lasted 3 minutes with a 1 minute rest period between intervals. Subjects completed up to 6 intervals. Once 6 intervals were comfortably completed, the speed was increased by 0.5 km/h in the following session to challenge the subject.

4.4.5 Rating of Perceived Exertion Scale

At the end of each interval, the subjects were asked to rate their perceived exertion, illustrated in figure 4.2. The scale values range from 6-20 with increases in perceived exertion described in words corresponding to higher values. The RPE for each training session was calculated as the maximal RPE reported at any given interval during the training session. Group mean RPE was calculated as the mean of this peak RPE given for any interval by each participant in the respective group.

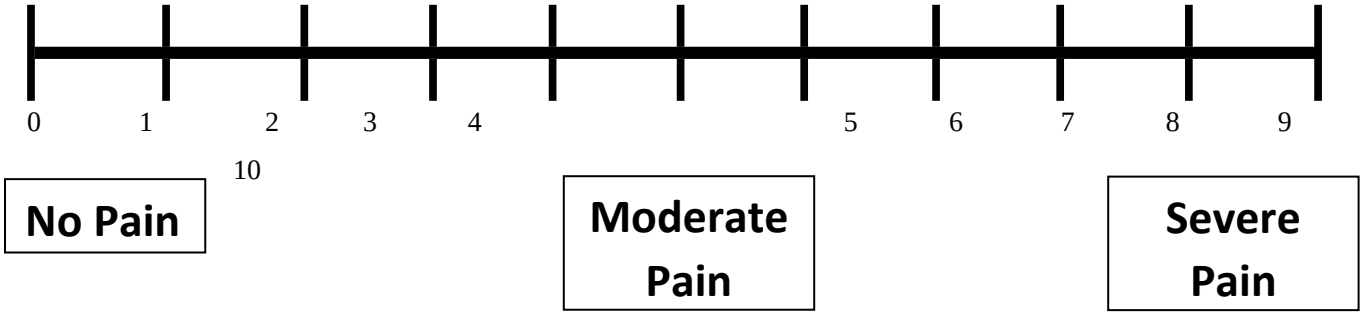
Table 4.2 Illustrating the Scale rate of perceived exertion

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

4.4.6 Perceived Muscle Soreness

Subjects were asked to rate their perceived muscle soreness on a modified delayed onset muscle soreness (DOMS) scale, shown in the image below. Values of 1-10 were presented to the subject, with 1 being minimal pain and 10 being unbearable pain. Subjects were asked to measure perceived muscle pain at baseline, immediately after, 6 hours and 24 hours after the 1st and the 10th training sessions.

Image illustrating 0–10 Visual Pain Rating Scale



4.5 Muscle Biopsy

No adverse effects were reported after muscle biopsies. Muscle biopsies were taken at baseline, and 6 hours after the 1st and the 10th HIIT sessions. For the downhill running group, an additional 2 muscle biopsies were taken in the overreaching phase of the study. These biopsies were taken at 6 hours after the last HIIT session and a resting biopsy after the training. In order to do multiple subjects in one visit from the medical Doctor, training schedules and biopsy schedules were tightly regulated in order to try to get the biopsy as close to 6 hours post exercise as possible. Due to slight delays in either the exercise session or the biopsy session, biopsies were taken between 5 and 6 and a half hours post exercise. The first biopsy was taken from the left leg, at the midpoint of the *vastus lateralis*. The second biopsy was taken from the right leg from the same point of the vastus lateralis by visual comparison done by the medical doctor experienced in this requirement for research studies. The third biopsy was taken from the left leg again, 1cm proximal to the previous biopsy site. This biopsy was taken 5 weeks after the first biopsy, allowing the biopsy wound to heal and any inflammation from the biopsy itself to subside before another biopsy was taken from the same leg. The additional biopsies taken from the runners in the downhill group were taken from the right leg and the 5th and final biopsy taken again from the left leg, again 1cm proximal to the previous biopsy sites.

4.5.1 The biopsy procedure

A licensed medical general practitioner (GP) with experience in percutaneous needle biopsies took the muscle biopsies for the study. The GP identified the biopsy site using the subject's arm length when he touched the lateral side of the *vastus lateralis*. Local anesthetic was then injected to the biopsy site using a dental needle, allowing a few minutes to ensure effect. If at any point the subject indicated pain, additional anesthetic was administered. A small incision was made with a scalpel blade, through the skin, dermis and muscle fascia. A 5 mm stainless steel biopsy needle (Stille, Sweden) was inserted into the incision. An assistant attached a suction device to the end of the needle and applied suction. This allowed a small portion of the muscle to be sucked into the needle. At this time, the GP. made 2 to 3 cuts with the biopsy needle, rotating the needle on its axis with every cut to ensure a smooth piece of muscle sample. 100-150 μg of muscle sample was obtained with each biopsy. The GP removed the needle, containing the muscle sample, wiped away any bleeding from the wound and applied Sterri strips in a star formation to close the wound. A Tegaderm plaster was

placed over the Sterri strips and finally a slightly elasticised Band-Aid was tightly wrapped around the leg ensuring pressure. The subjects were instructed by the GP to take the Band-Aid off 1 to 2 hours post biopsy and allow the plasters to naturally remove over time. If there were any abnormalities with healing or severe pain post biopsy, the subjects were instructed communicate with the investigator or the GP, who they help accordingly.

The muscle biopsy was quickly removed from the biopsy needle, dabbed with cotton wool to remove the blood and other fluids, placed in tissue mounting media (Tissue Tek, Leica, Germany) and frozen in liquid nitrogen cooled iso-pentane (see full freezing protocol in Appendix C). All samples were stored at -80° C.

4.6 Blood draw

A total of 11 blood draws were taken from the subjects over the course of the study as described in the study timeline. A total of 25 ml of blood per blood draw was taken from the antecubital vein by a certified phlebotomist. A 20 ml sample of whole blood was collected in EDTA-coated tubes (Ethylenediaminetetraacetic acid Vacutainer, BD) for subsequent laboratory analysis. A 5 ml sample of blood was collected in a serum separating tube (SST Vacutainer, BD). The blood samples were collected by staff of Pathcare Stellenbosch and analysed for circulating damage marker, creatine kinase, using their routine, standardized, automated method.

4.7 Muscle biopsy sample analysis

4.7.1 Sectioning

Muscle biopsies were slowly warmed from -80° C to -20° C and orientated to be sectioned in cross sections. For microcopy, cross sections at 12 µm thick were cut, using a cryostat (Leica CM1860 UV, Nussloch, Germany). The slides were coated with poly-L-lysine for the sample to adhere to the microscope slide. Six sections were sectioned per slide. For biochemical analysis, cryosections were sectioned at 40 µm and collected in a 2 ml Eppendorf tube, until a final weight of between 20 and 30 milligrams of sample. Samples were stored on ice for immediate homogenising.

4.7.2 Fluorescence staining

4.7.2.1 Basic Staining Protocol

Cryosections were thawed at room temperature for 10 minutes and placed in a humidifier box for the staining procedure. Sections were fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), pH 7.4, for 8 minutes. Sections were washed with 1X phosphate-buffered saline and Tween (PBS-T) for 5 minutes, 3 times. All wash steps were the same unless stated otherwise. Sections were blocked with 4 % Normal Donkey Serum (Abcam, ab7475, Cambridge, USA), 1 % Bovine Serum Albumin (BSA) (Roche, 70331927, Mannheim, Germany), 0.1 % Triton X-100 (BDH, 1014224, Poole, England) and sodium azide for 1,5 hours at room temperature in order to limit non-specific binding of primary antibodies. Sections were then incubated in primary antibody overnight at 4 degrees Celsius in a humidified box. Washed sections were incubated with corresponding conjugated secondary antibodies for 1 hour at room temperature. Antibody details can be found in the table 4.3 below. Following a wash step, nuclei were stained for 8 minutes with Hoechst dye (1:1000) before a final wash and mounting with a coverslip, using fluorescence mounting media (Dako, S3023, Carpentaria, USA).

4.7.2.2 Satellite Cell, capillary, MHCII, basal lamina, nuclear stain protocol

Due to the potential cross reactivity between the selection of antibodies available to the researcher, a modified protocol was administered to ensure optimal positive signal of all targets. Sections were stained with Pax7 primary antibody diluted in BSA and incubated overnight at 4 degrees C. Samples were washed with PBS-T 3 times for 5 minutes per wash and incubated with the secondary antibody diluted in BSA for 2 hours at room temperature. Following a wash step the samples were re-fixed in 4 % PFA for 15 minutes, washed again and incubated with the primary antibodies for CD31 diluted in BSA overnight at 4 degrees C. Samples were washed again as before, and incubated with the accompanying secondary antibody in BSA for 3 hours at room temperature. The samples were washed and re-fixed in 4 % PFA for 15 minutes before another wash step. Primary antibody for MHCII was diluted in BSA and incubated at 4 degrees C overnight. Samples were washed again and incubated in the accompanying secondary antibody diluted in BSA for 2 hours at room temperature. Samples were washed and incubated with wheat germ agglutinin (WGA) diluted in PBS for

10 minutes at room temperature before being washed again and incubated with Hoechst diluted in PBS for 8 minutes. Samples underwent a final wash in PBS and were dried out before coverslips were mounted using fluorescence mounting media. Full staining protocol see Appendix D.

4.7.3 Antibodies

Table 4.3: Showing the Primary Antibodies raised against their targets and the accompanying secondary antibody used to obtain positive signal

Primary Antibody	Company and catalogue number	Species	Dilution	Accompanying secondary antibody
MHCII	Hybridoma bank	Mouse	1:250	Abcam Donkey Anti-mouse Alexafluor- 488
Dystrophin	Abcam	Rabbit	1:250	Abcam Donkey Anti-rabbit Alexafluor- 594 (1:500, ab150064, Cambridge, USA)
Pax7	Hybridoma bank	Mouse	1:100	Abcam Donkey Anti-mouse Alexafluor- 555 (1:500, ab150110, Cambridge, USA)
CD31	Abcam (ab9498, Cambridge, USA)	Mouse	1:100	Abcam Donkey Anti-mouse Alexafluor- 647
Wheat Germ Agglutinin	Invitrogen (W1126, Eugene, USA)	Glycoprotein and cell surface dye	1:2000	Conjugated with Alexafluor-488
Hoechst		Nuclear dye	1:10 000	

4.7.4 Microscopy

Fluorescent imaging was achieved using a confocal microscope (Zeiss LSM 780, Germany). Settings were optimised using positive and negative controls (see appendix) in order to eliminate non-specific fluorescence ensuring the signal received by the samples was indeed true signal. Samples were visualised using a 20X objective (EC Plan-Neofluar 10x/0.30 M27) and a 40X objective (LD Plan-Neofluar 40x/0.6 Korr M27). The microscope is equipped with a GaAsp detector for detection of fluorescence. Images were analysed using imaging software (Zen Black 2011, Zeiss) and ImageJ software (National Institutes of Health, USA)

4.7.5 Image Analysis

4.7.5.1 Fibre type and cross-sectional area:

Fibre types were distinguished by either being MHCII positive fibres, which was indicative of a type II fibre, or MHCII negative fibre, which conversely was indicative of a type I fibre. Dystrophin or WGA was used to identify the basal lamina of the fibre. CSA was analysed for both type I and II fibres in all muscle biopsies across both groups.

4.7.5.2 Myonuclear identification:

Myonuclei were identified as positive Hoechst signal, and nuclei that are localized within the muscle fibre but almost exclusively at the periphery. Other nuclei were considered as interstitial nuclei and not counted. Unless situated in the centre of the fibre, then counted as a central nuclei. The number of myonuclei per fibre was analysed by counting the number of myonuclei/ number of fibres. In order to calculate the myonuclear domain, the CSA was divided by the number of myonuclei/fibre.

4.7.5.3 Satellite cell identification:

Satellite cells were identified if they met the following criteria:

1) Pax7 positive signal
2) Hoechst positive signal
3) Situated between the basal lamina and the sarcolemma

SC data were expressed as SC/fibre = Total SC pool size. Fibre type specific SC were identified with the above criteria, but with addition of the identification of the fibre type of the fibre where the SC was situated. Fibre type specific SC data were expressed as:

1) Type I SC/Type I Fibre = Type I SC pool size
2) Type II SC/Type II Fibre = Type II SC pool size

4.7.5.4 Capillary identification:

Capillaries were identified with endothelial marker, CD31 positive signal, forming a dot like shape situated between muscle fibres. Capillary to fibre ratio = total capillary number/total muscle fibre number.

4.7.5.5 Distance between Satellite Cells and Capillaries:

In order to assess the potential relationship between SCs and capillaries, the distance was measured between an identified SC and its nearest capillary, along the basal lamina.

4.7.5.6 Eliminating false positive signal and false identification:

The primary antibodies for Pax7, CD31 and MHCII were all raised in the same species. In order to overcome potential signal cross reactivity, the following analysis procedure was used. Re-fixing the sample in between steps could fix the antibody to its epitope. Pax7 antibodies were incubated first. Upon visualization, SCs were analyzed according to the above criteria and marked. Next, capillaries were identified. CD31 positive signal was identified as a capillary, but if there was CD31 positive signal colocalized with Pax7 positive signal it was not considered as a capillary but rather as a SC. Lastly MHCII positive fibres were identified, staining for MHCII is robust and therefore easy to identify.

At least 200 muscle fibres were analysed per subject, per biopsy.

4.8 Biochemical analysis of protein levels

4.8.1 Sample preparation

Approximately 20 – 30 mg of each sample was sectioned and placed in 2 ml capped tubes (Eppendorf, Germany). Samples were homogenized in 700 µl radioimmunoprecipitation assay (RIPA)-based lysis buffer (2X Complete Protease Inhibitor, 11697498001, Roche; 1X Complete Phosphatase Inhibitor, 4906845001, Roche, Switzerland) using a manual dispenser (PolyTron, Kinematica AG, Switzerland). Samples were then centrifuged for 3 minutes at 10 000 g. The supernatant was removed and used without further dilution or concentration as the final sample for analysis of selected constituents. Protein concentrations were determined with a commercial Bicinchoninic acid (BCA) kit (BCA protein assay, 23225, Thermo Fischer Scientific, USA) according to the manufacturer's guidelines.

4.8.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

20 µg of protein from each sample was used for electrophoresis on hand-cast polyacrylamide gels consisting of 12% separating gel and a 4% stacking gel (0.125 M Tris-HCl (pH 6.8), 12.5% Acrylamide, 1% SDS, 1% APS, 0.1% TEMED). Each gel contained the protein ladder in the first lane, a reference sample in the second lane and all 3 samples of each volunteer (Baseline, Acute and 4 Weeks). A total of 3 gels were run for each protein assessed. The reference sample contained protein lysate from one of the subjects, with the same reference sample used in all the gels enabling the comparison of protein expression between the gels to be calculated accurately. Prior to sample loading, tissue lysates were mixed with 3 µl of Laemmli solution (S3401, Sigma-Aldrich, USA) containing 5% β-mercaptoethanol (4162100, Unilab, South Africa) and boiled for 5 minutes at 95°C. A Precision Plus Protein™ Kaleidoscope™ marker (161-0375, Bio-Rad, USA) was used to obtain protein band sizes. Electrophoresis was performed on a small size system (Mini-PROTEAN® Tetra Cell, Bio-Rad, USA) at120V, for a duration of roughly 120 minutes or just before the protein front ran off the gel.

4.8.3 Western blotting

Post electrophoresis, gels were transferred onto a nitrocellulose membrane (RPN 3032D, GE Healthcare, Life science, UK) via a Turbo-blot transfer system (Bio-Rad, USA). Blots were transferred at 25V and 2.0A for 20 minutes. To confirm successful transfer of proteins onto the membrane, post transfer blots were stained with Ponceau S and imaged (ChemidocMP, Bio-Rad, USA) that was supported by Image lab software 4.0 (Bio-Rad, USA). After blocking for 1.5s hour with 5% fat free milk powder (Parmalat, South Africa) in TBS-T, membranes were incubated with primary antibodies for MyoD (sc32758, Santa Cruz Biotechnology, USA) and Myogenin (M3559, DAKO, USA), in 5% milk powder in TBS-T at 4^{o}C overnight. After washing, membranes were incubated with an anti-mouse secondary antibody conjugated with HRP (7076, Cell Signalling, USA) at room temperature for 2 hours. The membranes were washed and imaged with the used of enhanced chemiluminescence (ECL, SuperSignal West Femto Maximum Sensitivity Substrate, 34094, Thermo Scientific, USA). Membranes were subsequently imaged using the Chemidoc MP and Image Lab software (Bio-Rad, USA). Immunoreactive proteins were subsequently quantified using the software and were normalised against total protein from Ponceau staining as well as normalised to the reference sample. Full protocol as well as information regarding gel percentage, voltage of electrophoresis, and antibody concentrations can be found Appendix E.

4.9 Statistical Analysis

All data are represented as the Mean ± Standard Deviation (SD). To analyse the performance data, CK activity and PMP, a mixed model ANOVA was used with time as the within subject factors and group as the between subject factor. Muscle fibre analysis was done using a mixed model ANOVA with time and fibre type as within subject factors and group as the between subject factors. A Fischer LSD post hoc test was performed to correct for multiple testing. A p value $p < 0.05$ was considered significant.

Chapter 5: Results

5.1 Group Characteristics

When comparing the two groups, subjects were of similar age, height and weight (21 ± 1.0 years UHG vs 21 ± 1.5 years DHG; 175 ± 6 cm UHG vs. 180 ± 8 cm DHG; 73 ± 5 kg UHG vs. 77 ± 12 kg DHG). No significant differences in baseline performance results were apparent between the groups for VO_2max or road 5 km time trial time (see table 5.1). The UHG had significantly higher baseline maximal isometric force production compared to the DHG (27% higher).

Table 5.1 Subject Characteristics

	UPHILL	DOWNHILL
VO_2max (ml/kg/min)	59.48 ± 4.3	59.08 ± 4.7
Isometric strength (N)	934 ± 95	734 ± 133
5 km time trial time (min:sec)	22:05 ± 0:42	22:50 ± 0:59

Data expressed as Mean ± SD.

5.2 Training Intervention

One subject from the UHG withdrew from the study before the training phase of the study took place due to unrelated back injury, but the 5 other subjects in the UHG completed the training. All 6 subjects in the DHG completed the 4-week HIIT protocol. Subjects from the UHG had difficulty finishing 6 intervals per session, unlike the DHG for which every subject completed all 6 intervals at every training session. The UHG trained at a slower speed per interval compared to the DHG ($p < 0.01$; for means and SDs see table 5.2), which was expected due to the gradient difference. Due to the slower speed and a smaller number of intervals per session, the UHG ran a shorter distance per session compared with the DHG and therefore covered a total distance less than the DHG ($p < 0.05$) over the 4 weeks of HIIT. For the HIIT indoor training distances see table 5.2. RPE was reported at the end of each interval of each session and the highest RPE for any interval of a particular session was recorded and the mean for the 10 HIIT sessions was used per runner to calculate the mean for the group for inter-group comparison (for mean and SDs, see table 5.2). RPE was significantly higher for the subjects in the UHG $p < 0.001$) compared to the DHG. As more HIIT sessions were done the UHG subjects were able to complete more intervals per session.

The calculation of between-group difference for RPE was therefore also done for only the sessions that all the intervals were completed (see figure 5.2.1). Even when they completed 6 intervals per session the RPE was higher for those UHG than the DHG ($p < 0.05$).

Table 5.2 4 Week HIIT protocol for uphill and downhill running

	UPHILL	**DOWNHILL**	**p value**
Number of sessions	10	10	
Intervals	53 ± 0.10	$60 ± 0	≤ 0.0005
Speed (km/h)	13.5 ± 0.2	$17.1 ± 0.4	0.0012
Distance per session (km)	3.1 ± 0.2	$5.1 ± 0.1	0.0003
Total distance (km)	31.9 ± 1.8	$51.2 ± 1.3	0.0022
RPE	19.5 ± 0.3	$16.3 ± 0.6	0.0011

Data expressed as Mean ± SD.
$ Significance between groups

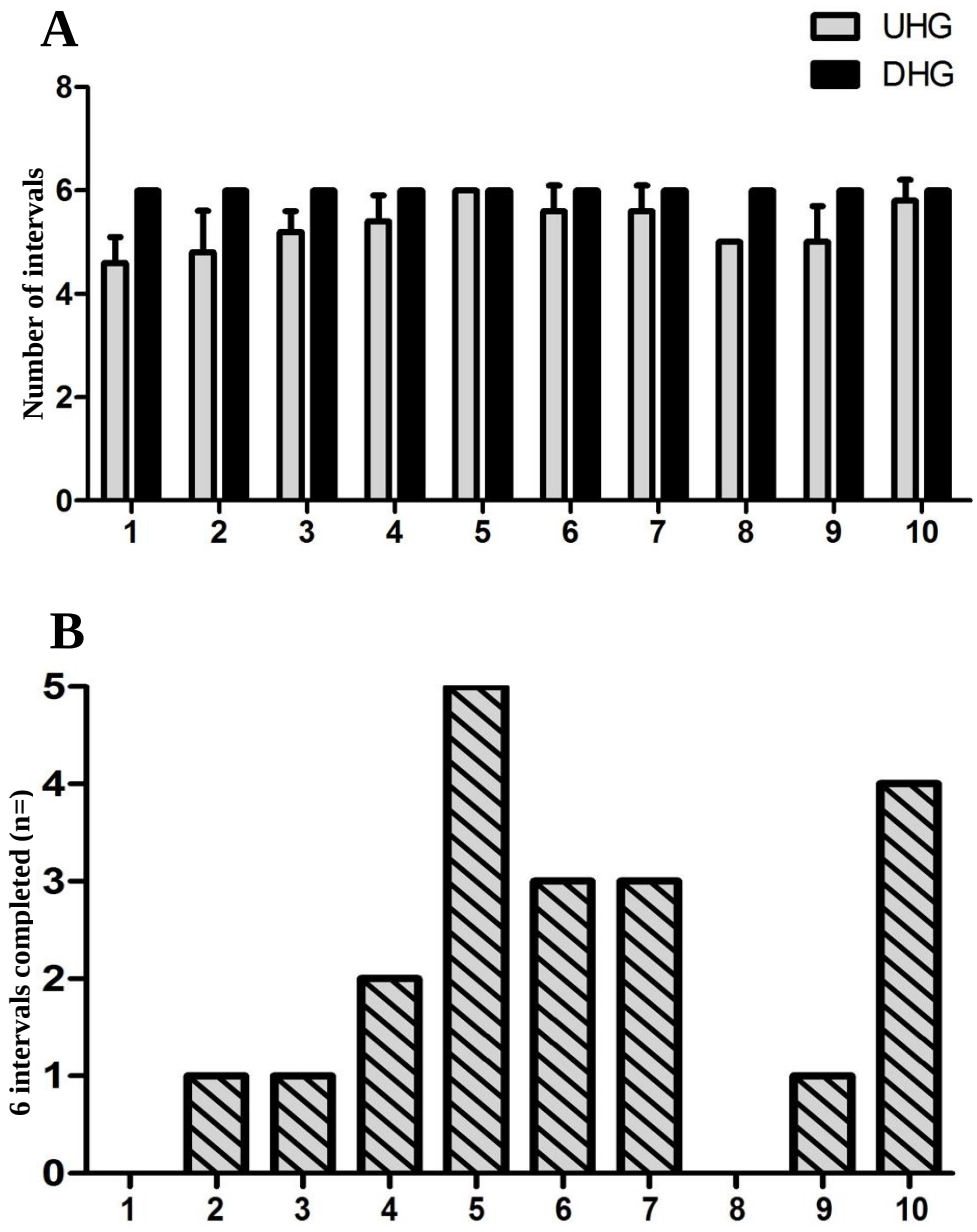


Figure 5.2.1 Intervals completed during the HIIT session

A: The number of intervals completed for all 10 HIIT sessions for the UHG (in grey) and DHG (in black).

B: The number of subjects in the UHG that completed 6 intervals during the 10 HIIT session.

The heart rate responses of a single HIIT session were measured using heart rate monitors. HR was successfully recorded without interruption for the entire session for 4 subjects in the UHG and 4 subjects in the DHG (n=4). Subjects from both the UHG and DHG had similar heart rate responses to the training session. There were no significant differences between the groups for average heart rate during the session. The heart rate responses to a HIIT session are shown in Figure 5.2.2, including an indication of the number of subjects who completed each interval in each group.

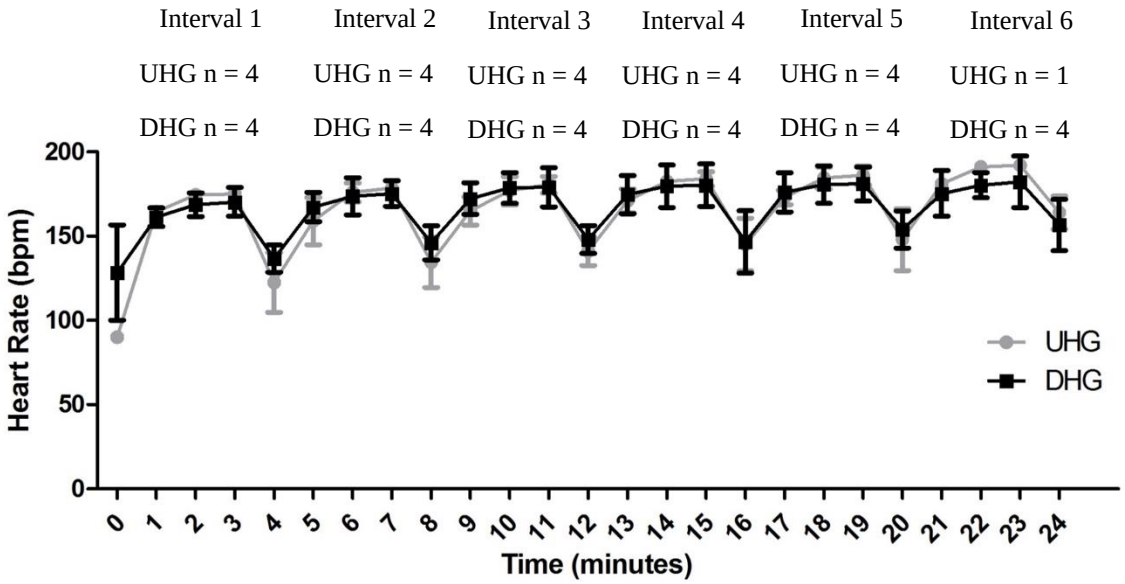


Figure 5.2.2 Heart Rate Response to a high intensity interval session
The heart rate response to an individual session of participants from the UHG (n=4) in grey and the DHG (n=4) in black. Data are expressed as Mean ± SD

5.3 Training load

Subjects were instructed to maintain their training status and training type, to ensure any performance and muscular adaptation was due to the HIIT laboratory running protocol and not due to any changes they chose to include in their external personal training load. The training load 3 weeks prior to the introduction of the 4 week HIIT protocol is expressed in hours per week, since the majority of training was of multiple types (mainly social soccer or indoor cycle spinning classes; but also 2 subjects participating in social field hockey and 2 doing recreational gymnasium exercises). This training load was maintained by the subjects throughout the training phase (see table 5.3). Therefore, the difference in training load during the laboratory HIIT phase of the study was due to the additional HIIT sessions every week and not the addition of external forms of training or changes in duration of personal external training (Figure 5.3). When including the HIIT sessions, there was a significant difference between running volume before HIIT and during HIIT when running volume was expressed as distance per week, since the DHG ran further per session compared to the UHG (Figure 5.3).

Table 5.3 Training load prior and during training

	Before HIIT	**During HIIT***
Training volume (hrs/week)	2.7 ± 0.6	2.7 ± 0.6
No. of workouts (w/week)	3.4 ± 0.5	3.3 ± 0.5
No. of running sessions per week	2.3 ± 0.4	2.0 ± 0,5
Running volume (km/week)	9.2 ± 1.2	8.4 ± 1.7
Running volume (hrs/week)	1.5 ± 0.3	1.2 ± 0.4

Data expressed as Mean ± SD.
* Training load excluding the laboratory HIIT

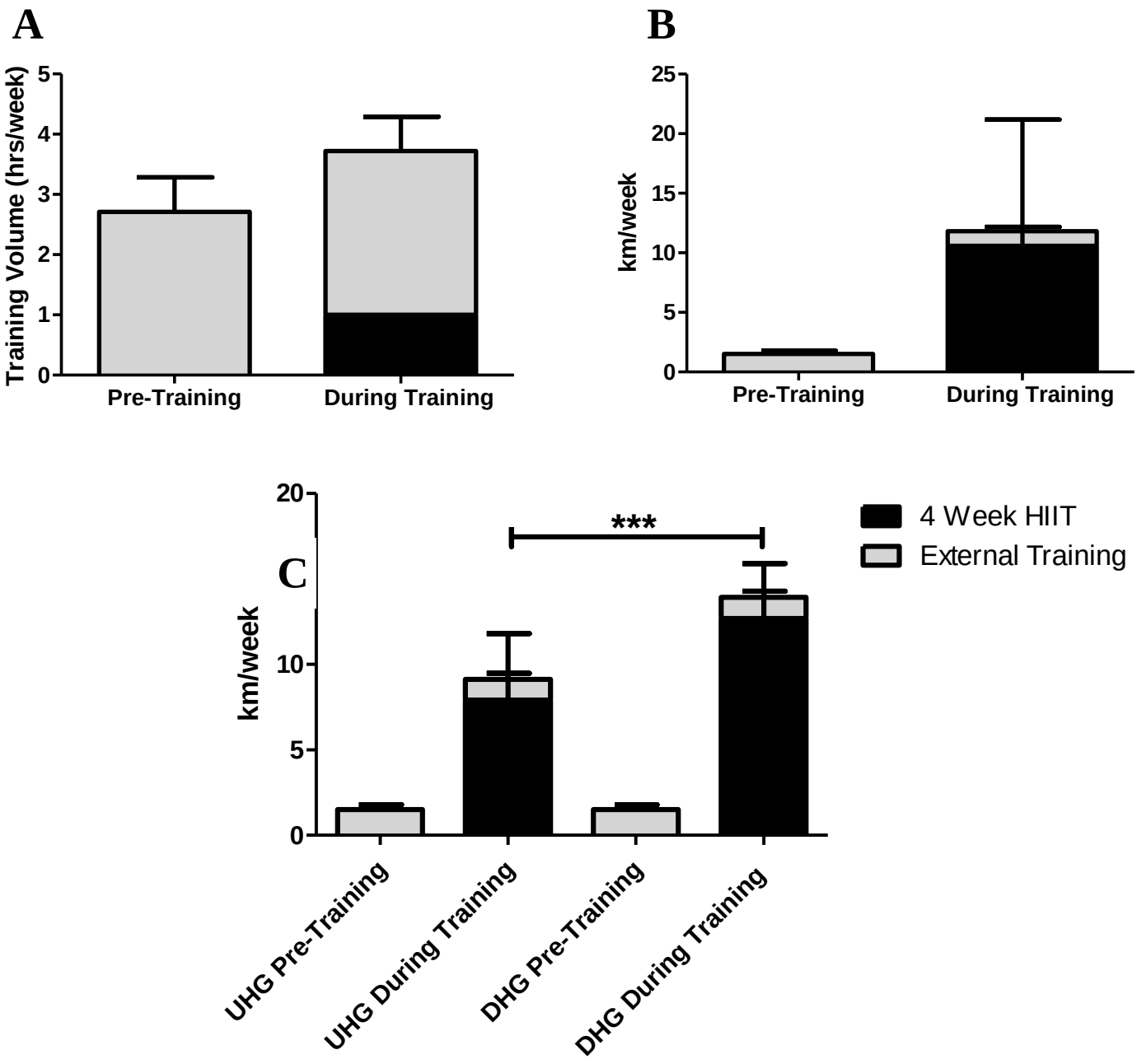


Figure 5.3. Training volume before and during training.

A: Training Volume in hours/week of all participants (n=11). Training volume from the HIIT protocol in black, and external training in grey.

B: Training volume in km/week of all participants (n=11). Training volume from the HIIT protocol in black, and external training in grey.

C: Training volume of the UHG (n=5) and the DHG (n=6), before and during training. Running volume from the 4 week HIIT protocol displayed in black and external running volume in grey.

Data are expressed as Mean ± SD. *** Significantly different compared to the other group

5.4 Perceived Muscle Pain

Perceived muscle pain (PMP) was subjective data given by the subjects and was obtained immediately after, 6 hours and 24 hours after the 1st and the 10th session of the HIIT protocol. The DHG showed a significant increase in PMP 24 hours after the 1st exercise bout ($p < 0.05$) compared to the other immediately and 6 hours after the training session. After the 4 weeks of HIIT there was a significant decrease in perceived muscle pain immediately after, 6 and 24 hours after training compared to 24 hours after the 1st training session. There was no difference in PMP between immediately after, 6 hours and 24 hours after the 10th session. The UHG showed no significant increase in PMP over all the time points for both the 1st and 10th session (Figure 5.4).

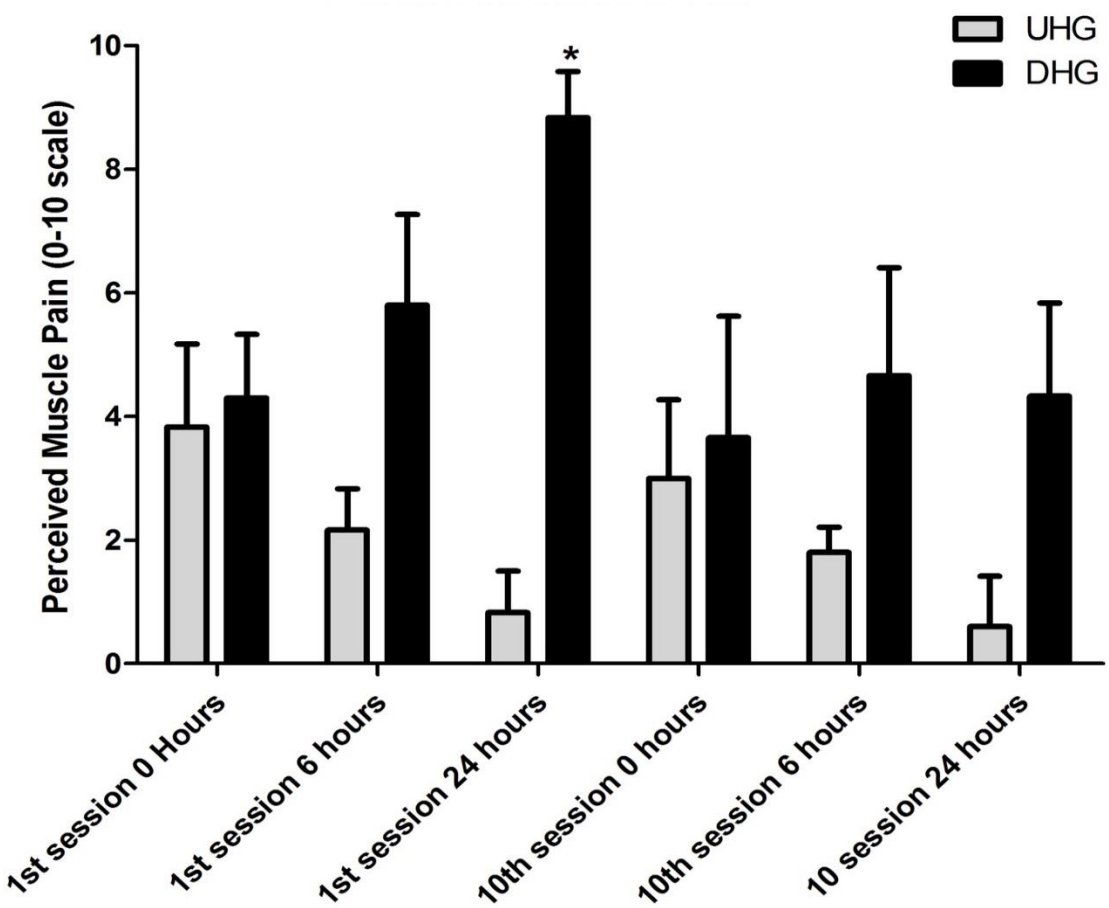


Figure 5.4 Perceived muscle pain immediately after, 6 and 10 hours after the 1st and the 10th exercise session.

Perceived muscle pain of subjects in the DHG (n=6) in black and the UHG in grey (n=5) immediately after, 6 hours and 24 hours after the 1st and the 10th HIIT session.
Data are expressed as Mean ± SD. * Significantly different from other time points ($p < 0.05$).

5.5 Performance changes after 4 weeks HIIT

The UHG significantly improvement VO_2max ($p < 0.05$; see table 5.5) and PTS ($p < 0.01$; see figure 5.5.1) with 4 weeks of HIIT whilst the DHG did not. In contrast, the DHG had a 25% improvement in quadriceps muscle strength ($p < 0.05$, for absolute values see table 5.5.2), whilst the UHG showed no improvement with training. Data for isometric strength relative to body mass (N/kg) are presented in figure 5.5.2. Both the UHG and the DHG had similar improvement in outdoor road 5km time trial performance (3.5 ± 1.1% UHG: 3.5 ± 1.6% DHG, $p < 0.05$) with 4 weeks of HIIT. For time to complete the course and for running pace, see Table 5.5.

Table 5.5 Changes in performance after 4 weeks HIIT

	UPHILL			DOWNHILL		
	BASELINE	**4 WEEKS**	**% CHANGE**	**BASELINE**	**4 WEEKS**	**% CHANGE**
Laboratory Testing						
VO_2max (ml/kg/min)	59.5 ± 4.3	*61.9 ± 3.1	4.2 ± 2.6	59.1 ± 4.7	59.5 ± 4.9	0.7 ± 1.2
Isometric force (N)	934 ± 95	893 ± 100	-4 ± 3.6	734 ± 133	* 893 ± 55	25 ± 22
Outdoor Testing						
5km TT time (min:sec)	22:05 ± 0:42	*21:16 ± 0:55	3.5 ± 1.1	22:50 ± 0:59	*22:02 ± 1:31	3.5 ± 1.6
5km TT pace (min:sec/km)	4:30 ± 16	*4:15 ± 10	3.5 ± 1.1	4:33 ± 11	*4:24 ± 18	3.5 ± 1.6

Data are expressed as Mean ± SD
* Significance due to training $p < 0.05$

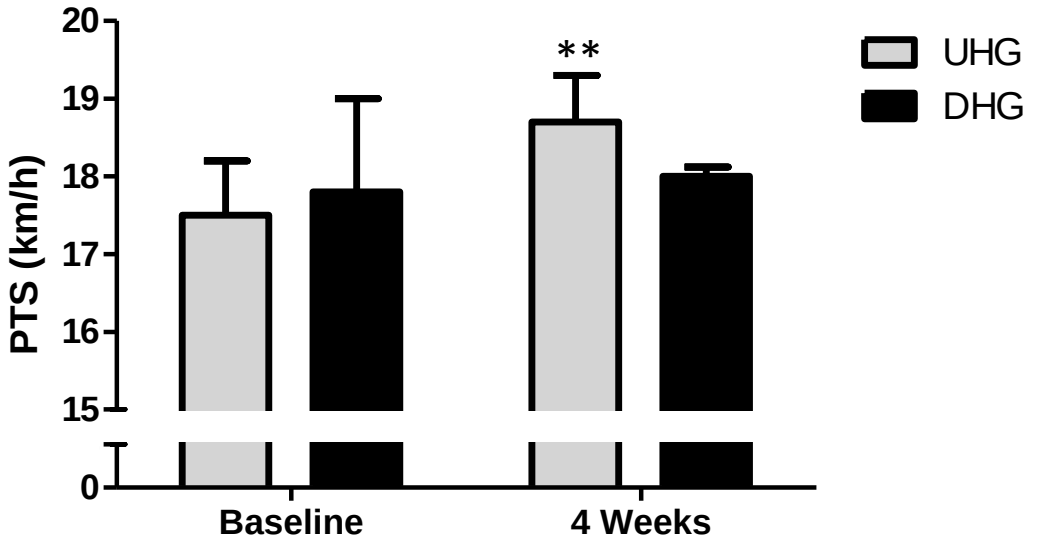


Figure 5.5.1 Flat treadmill incremental exercise test to exhaustion: Peak treadmill speed at baseline and after 4 weeks of downhill or uphill HIIT

PTS (km/h) of all subjects in the DHG in black (n=6) and the UHG in grey (n=5).
Data are expressed as Mean ± SD. ** Significantly different from baseline ($p < 0.01$).

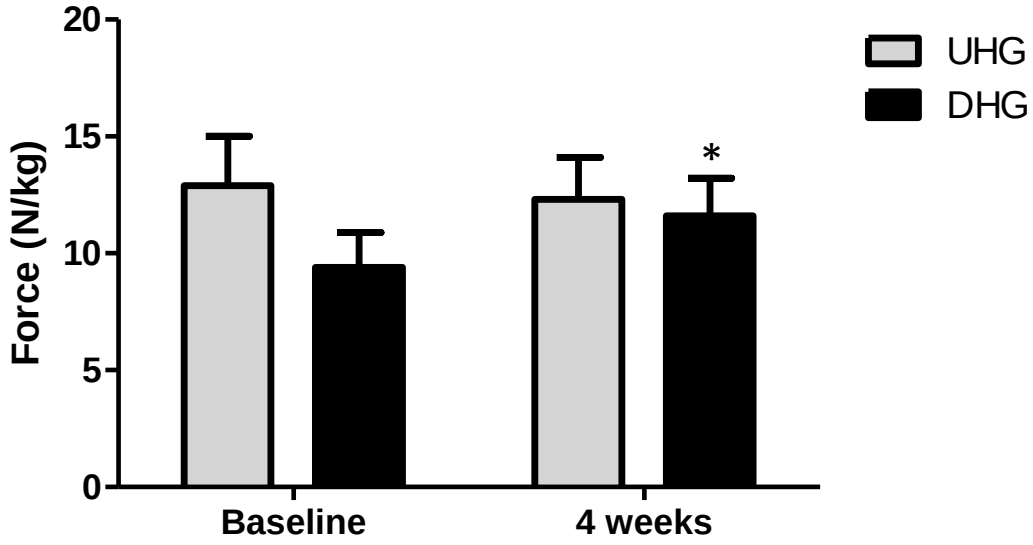


Figure 5.5.2 Maximal isometric force before and after HIIT in the downhill and uphill HIIT groups

A: Maximal isometric force of all subjects in the DHG in black (n=6) and the UHG in grey (n=5).
B: Maximal isometric force per body weight of all subjects in the UHG (n=5) in grey and the DHG (n=6) in black.
Data are expressed as Mean ± SD. * Significantly different from baseline ($p < 0.05$)

5.6 Indirect Marker of muscle damage

Serum creatine kinase activity was measured and presented in table 5.6. For analysis of results the CK activity was measured as a percentage change from the baseline. CK activities for each participant in the DHG (n=6), and is shown in figure 5.6.1

Table 5.6 The CK levels during the 4 week HIIT

	Baseline	Week 1 Pre	Week 1 Post	Week 2 Pre	Week 2 Post	Week 3 Pre	Week 3 Post	Week 4 Pre	Week 4 Post
UHG	186 ± 94	204 ± 109	293 ± 178	209 ± 115	255 ± 140	199 ± 86	248 ± 114	242 ± 217	300 ± 270
DHG	172 ± 97	171 ± 72	*328 ± 80	216 ± 151	*296 ± 158	200 ± 90	*278 ± 84	201 ± 100	*283 ± 95

Values at each time point represent CK (IU/L)
Data are expressed as Mean ± SD
* Significant increase from baseline

CK values were normalized to a percentage change from baseline to be illustrated graphically.

Pre exercise in the UHG, there was no significant change in CK activity at the time points over the 4 weeks of HIIT compared to baseline (0% change Baseline vs. 9 ± 44% change Week 1 Pre-HIIT vs. 11 ± 29% change Week 2 Pre-HIIT vs. 6 ± 40% change Week 3 Pre-HIIT vs. 23 ± 58% change Week 4 Pre-HIIT). There was no significant difference between the 6 hour post exercise time points compared to baseline throughout the 4 weeks of HIIT (0% change Baseline vs. 36 ± 49% change Week 1 Post-HIIT vs. 26 ± 30% change Week 2 Post-HIIT vs. 24 ± 40% change Week 3 Post-HIIT vs. 38 ± 46% change 4 Weeks Post-HIIT).

In the DHG, there was no significant difference in the change in creatine kinase activity between Baseline and the pre-exercise time points over the 4 weeks of HIIT (0% change Baseline vs. 5 ± 52% change Week 1 Pre-HIIT vs. 25 ± 77% change Week 2 Pre-HIIT vs. 15 ± 26% change Week 3 Pre-HIIT vs. 16 ± 65% change Week 4 Pre-HIIT). There was a significant difference between baseline and 6 hours after the first exercise bout (0% change Baseline vs. 90 ± 91% change Week 1 Post-HIIT, $p > 0.01$). The same was shown for baseline compared to the other time points 6 hours after exercise over the 4 weeks of HIIT

but to a lesser extent (0% change Baseline vs. 71 ± 67% change 2 weeks Post-HIIT vs. 61 ± 55% change 3 weeks post-HIIT, $p > 0.05$, vs. 63± 72% change 4 weeks post-HIIT $p > 0.05$).

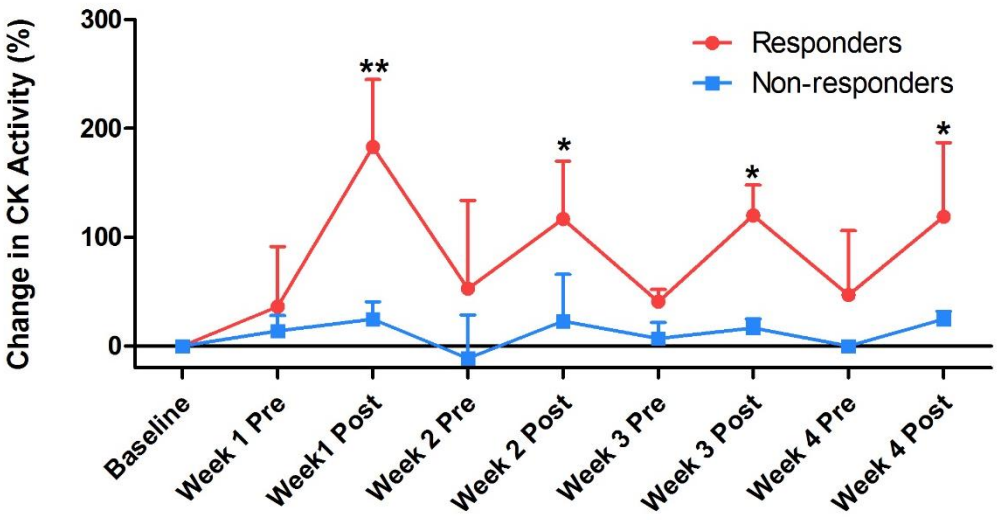


Figure 5.6.1 The change in creatine kinase activity for the downhill responders and non-responders in response to 4 weeks of HIIT

The percentage change of CK activity compared to baseline in the DHG responders (n=4) in red and non-responders (n=2) in blue.
Data are expressed as Mean ± SD. * Significantly different compared to baseline ($p < 0.05$). ** Significantly different compared to baseline ($p < 0.05$).

When comparing the change in CK activity over the 4 weeks of HIIT, there was no significant differences between the groups over all the pre-exercise CK activity time points. There was no significant difference between the groups at Week 1 post-HIIT and Week 2 post-HIIT (36 ± 49 % change UHG vs. 90 ± 91% change DHG Week 1 Post-HIIT, $p = 0.06$; 26 ± 30% change UHG vs. 71 ± 67% change DHG Week 1 Post-HIIT, $p = 0.06$). There was a significant difference between the groups at Week 3 Post-HIIT and Week 4 Post-HIIT (24 ± 40% change UHG vs. 61 ± 59% change 3 weeks HIIT DHG, $p = 0.04$; 38 ± 46 % change UHG vs. 63 ± 78% change DHG 4 Weeks Post-HIIT, $p = 0.04$).

The DHG was divided into CK responders (n=4) who responded to 1st bout of downhill running and non-responders (n=2) who did not show an increase in CK activity post the first session (Figure 5.6.1). The responders group had a significant increase in change in CK activity from baseline 6 hours after the 1st exercise session ($p < 0.01$) and 6 hours after the 1st session of week 2,3 and 4 ($p < 0.05$). The non-responders group had no significant change in CK activity from baseline CK activity over any of the time points (figure 5.6.1). The DHG responders was then compared to the UHG and there was a significant difference between the DHG responders and UHG 6 hours after the 1st bout of exercise and after the 3rd week (figure 5.6.2, $p < 0.05$)

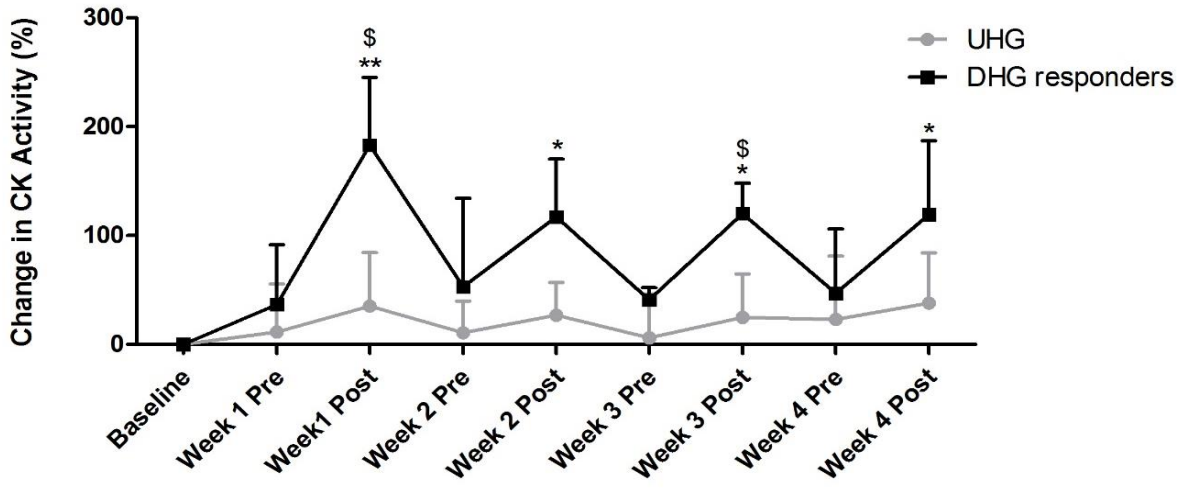


Figure 5.6.2 The change in creatine kinase activity for the downhill responders and the uphill group in response to 4 weeks of HIIT

The change in CK activity from baseline in all time points over the 4 week HIIT for the UHG (n=5) in grey and the DHG responders (n= 4) in black. Data are expressed as the mean percentage change from baseline. * Significant difference from baseline $p < 0.05$. ** Significant difference from baseline $p < 0.01$. $ Significant difference between groups $p < 0.05$.

The change in CK activity from baseline was correlated with perceived muscle pain at baseline, 6 hours after the first exercise session and 6 hours after the tenth exercise session, there was a significant correlation between the two ($r^2 = 0.56$, $p < 0.0001$, figure 5.6.3)

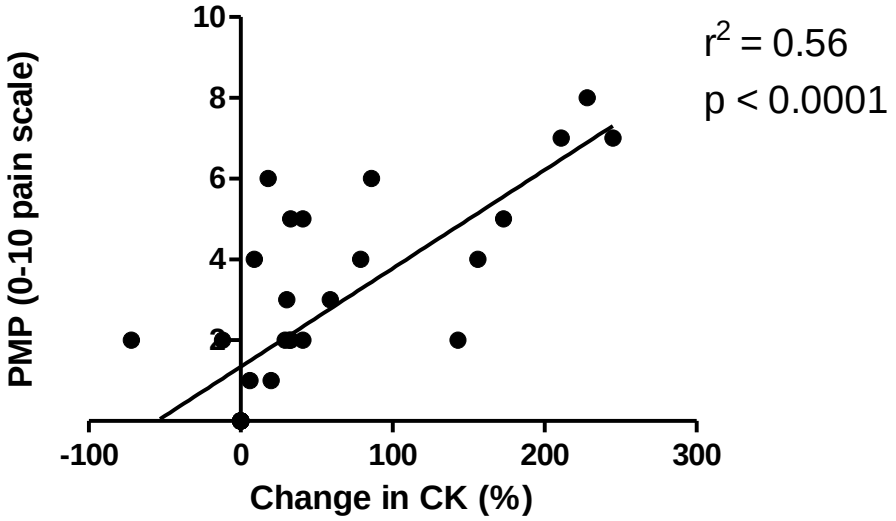


Figure 5.6.3 Correlation between perceived muscle pain and creatine kinase activity
Correlation between the PMP 6 hours after HIIT session 1 and CK values from blood drawn at the same time point, expressed in International Units per L of all participants (n=11)
C: Correlation between PMP and the percentage change in CK activity in all participants (n=11)

5.7 Central nuclei

Central nuclei are myonuclei that are situated within the muscle fibre and are indicative of muscle fibre damage or regeneration, or both. Central nuclei were illustrated with immunofluorescence after staining with WGA to identify the muscle fibre boarders, MHCII to determine muscle fibre type and Hoechst to identify nuclei (Figure 5.7.1).

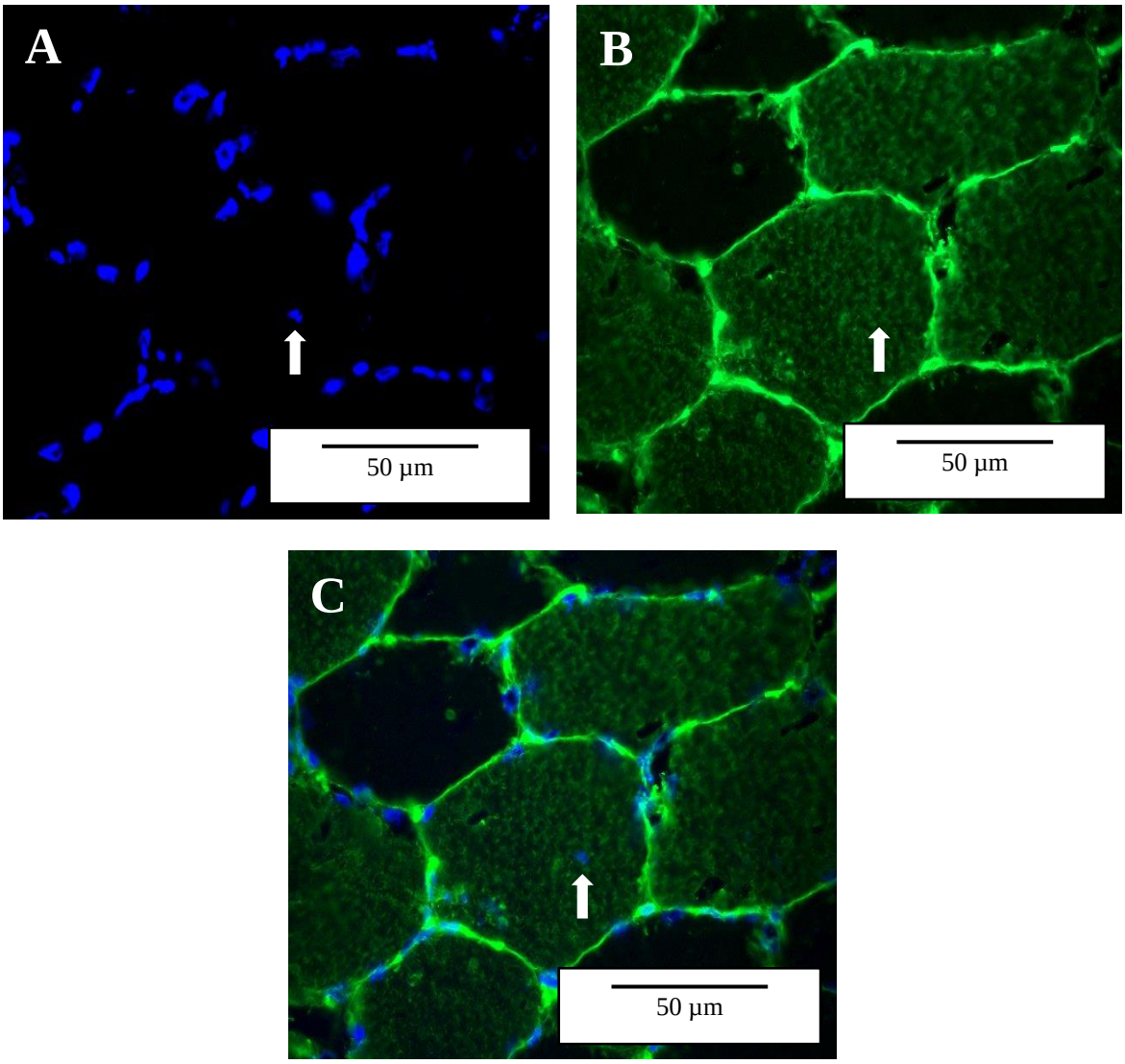


Figure 5.7.1 Central nuclei as a result of eccentric exercise-induced muscle damage
Representative single view channels A: Nuclei stained with Hoechst in blue, the white arrow highlighting a central nucleus within the muscle fibre B: Muscle fibre borders stained with WGA and fibre isoforms stained with MHCII in green C: Merged image illustrating the central nucleus situated within the muscle fibre borders of a type II muscle fibre. Magnification 400x. Scale bar 50µm

There were no central nuclei located in the UHG or the DHG biopsies at baseline in any of the fields of view assessed (refer to section 3.x for description of counting). The UHG had no significant increase in central nuclei 6 hours after the first bout of uphill running (0 Central nuclei/100 fibres Baseline vs. 1.2 ± 1.6 central nuclei /100 fibres Acute). After the 4 weeks of HIIT there was no significant increase in central nuclei compared to baseline (0 central nuclei/100 fibres Baseline vs. 0.9 ± 0.7 central nuclei/100 fibres 4 weeks) and compared to the acute time point (1.2 ± 1.6 central nuclei/100 fibres Acute vs. 0.9 ± 0.7 central nuclei/100 fibres 4 weeks). There was a significant increase in the number of central nuclei in the DHG 6 hours after the first bout of downhill running ($p < 0.001$ vs. Baselines and $p < 0.05$ between groups; see figure 5.7.2 Panel A). The range for the DHG participants was from minimum of 3 nuclei per100 fibres to 14 nuclei per 100 fibres. This was irrespective of the fibre type in which the central nucleus was observed. After 4 weeks of HIIT there was a significant decrease in the number of central nuclei compared to the acute time point (8.9 ± 5.6 central nuclei/100 fibres Acute vs. 0.4 ± 1 central nuclei/100 fibres 4 weeks; $p < 0.001$). There was a significant difference between the UHG and the DHG at the acute time point but not at baseline nor after 4 weeks of HIIT. Closer analysis of the central nuclei in the DHG showed that there were more central nuclei at the acute time point in type II muscle fibres compared to type I fibres ($p < 0.001$; see figure 5.7.2 Panel B).

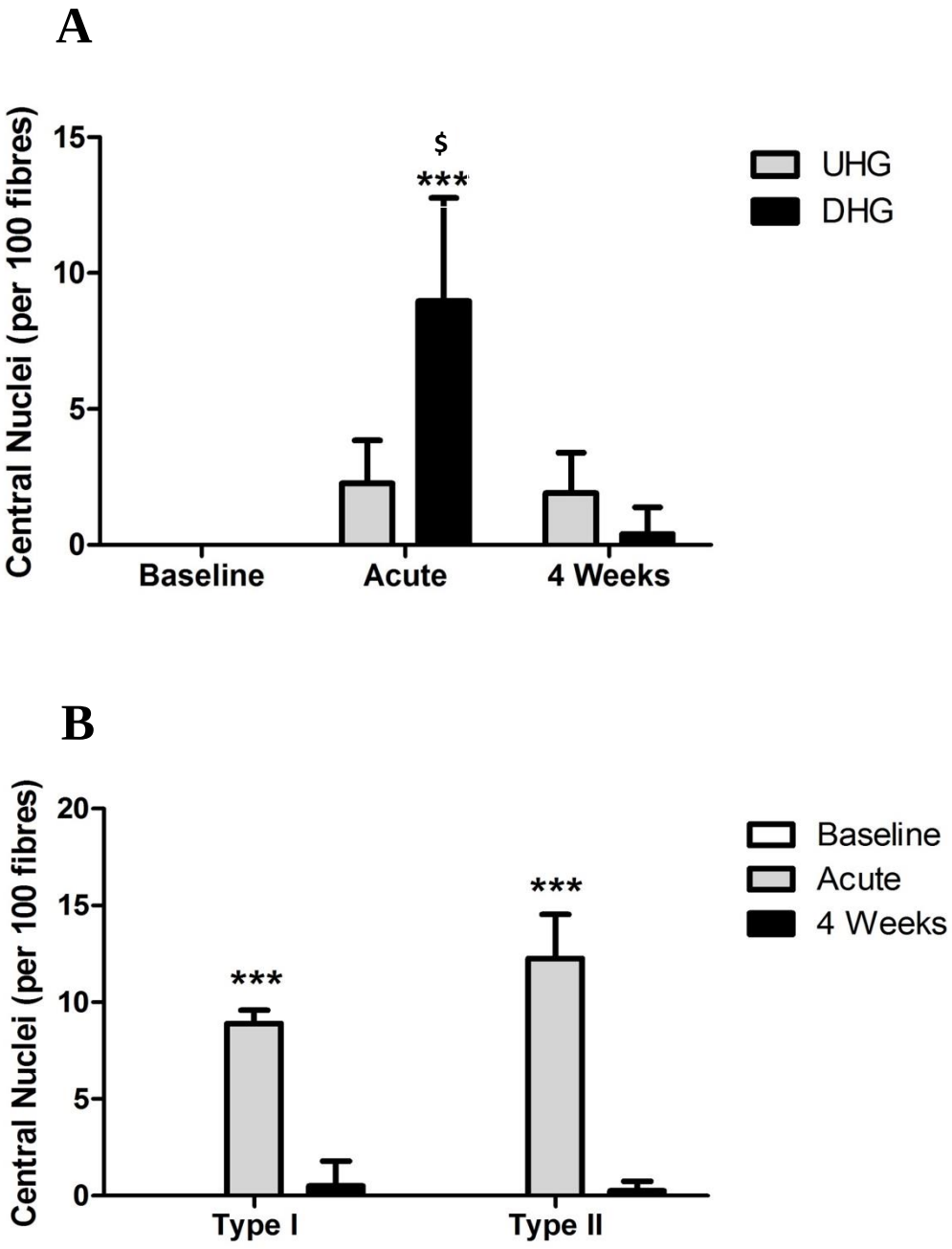


Figure 5.7.2 Central nuclei assessed for all fibre types and specific fibre types

A: The number of central nuclei per 100 fibres in the UHG (n=5) in grey and the DHG (n=6) in black at baseline, acute and 4 weeks.

B: The number of central nuclei per 100 fibres in the DHG (n=6) type I and type II fibres at baseline, in white, acute, in grey, and 4 weeks, in black.

Data are expressed as mean ± SD.

*** Significantly different from other time points, $p < 0.001$.

$ Significantly different between groups, $p < 0.05$

The change in the number of central nuclei/100 fibres from baseline tended to correlate with the change in CK activity at the acute time point. (See figure 5.7.3).

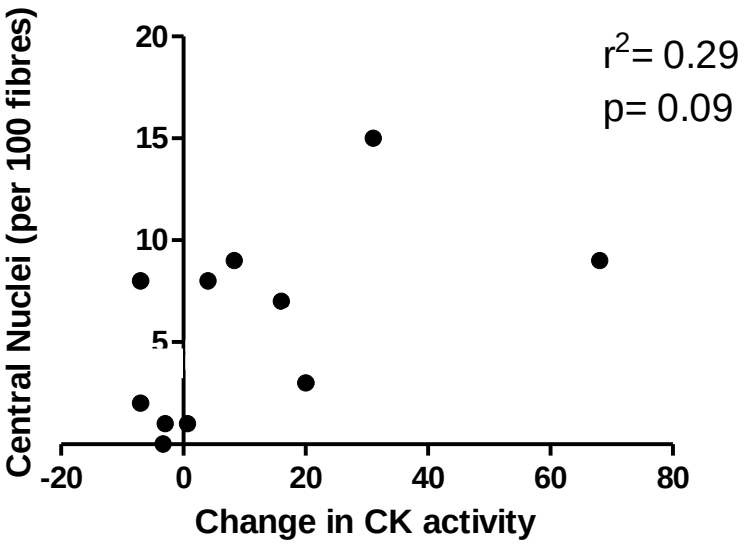


Figure 5.7.3 No correlation between central nuclei and creatine kinase activity
There is no correlation between the number of central nuclei after the first acute bout of HIIT vs. the change in CK activity (6 hr – baseline).

5.8 Muscle Fibre Morphology

Muscle fibre cross-sectional area and myonuclear content of both type I and type II fibres were determined based on immunofluorescence staining for MHCII to identify type II fibres, dystrophin to identify the muscle fibre borders and Hoechst to identify nuclei (Figure 5.8.1).

Fibre type

Fibre type proportion was calculated for each of the three muscle biopsies taken, for all subjects There was no significant difference between the groups for fibre type distribution (type I and type II) over the 4 weeks of training in the UHG (48 ± 7.6% type I and 52 ± 7.6% type II Baseline vs. 49 ± 10.1% type I and 51 ± 10.1% type II 4 weeks, $p = 0.42$) nor in the DHG (51 ± 15.8% type I and 49 ± 15.8% type II Baseline vs. 51 ± 9.6% type I and 49 ± 9.6% type II 4 weeks, $p = 0.27$). CK activity response at the acute time point were correlated with the percentage of type II fibres per subject. There was not a significant correlation ($r^2 = 0.32$, $p = 0.052$). The p value suggests that there is a statistical trend between the percentage of type II fibres and the CK response following exercise.

Fibre Cross Sectional Area

Muscle fibre cross-sectional area was significantly larger in type II fibres than type I fibres in both groups across all three time points (UHG Baseline $p = 0$, 4 weeks $p = 0$; DHG Baseline $p = 0$, 4 weeks $p = 0$). There was no significant increase in muscle fibre CSA in the UHG in either type I (5510 ± 2080 μm^2 Baseline vs. 6221 ± 1188 μm^2 4 Weeks, $p = 0.35$) or type II fibres (7804 ± 2030 μm^2 Baseline vs. 8238 ± 1958 μm^2 4 Weeks, $p = 0.56$, Figure 5.8.2 panel A). There was a significant increase in muscle CSA in the DHG in both type I fibres (6543 ± 2111 μm^2 Baseline vs. 8448 ± 1818 μm^2 4 Weeks, $p = 0.01$) and type II fibres (8342 μm^2 2629 μm^2 Baseline vs. 11103 ± 2495 μm^2 4 Weeks, $p = 0$) after 4 weeks of HIIT compared to the baseline (Figure 5.8.2 panel B). To determine possible fibre swelling response to the first HIIT session, CSA was calculated for the Acute biopsy. There was no difference between groups at baseline or at the acute time point, but after 4 weeks of HIIT the DHG had a significantly larger mean CSA compared to the UHG in both type I and type II fibres. Muscle

fibres were binned into groups differing in size by 1000 μm^2. Even though, more fibres were assessed in the 4 week biopsies for the UHG since it might be expected that more variation would occur after training, the size category frequency distribution of muscle fibre CSA showed no change in this group with 4 weeks of HIIT. In contrast, a shift in frequency distribution towards bigger CSA size categories (rightward shift) in the DHG was seen after 4 weeks of HIIT (Figure 5.8.3).

To understand the variability in the maximal isometric force production of the DHG (734 ± 133 N Baseline vs. 893 ± 55 N 4 Weeks, $p < 0.05$), the CSA was correlated with the maximal isometric force production at baseline and after 4 weeks of training (figure 5.8.4). There was a significant correlation between the change in CSA and the change in force production, expressed in Newton ($r^2 = 0.45$, $p = 0.02$) and a significant correlation between the change in CSA and the change in normalized force production, expressed as Newton per kg ($r^2 = 0.45$, $p = 0.02$).

Myonuclei content

The 4 weeks of HIIT did not increase the number of myonuclei per fibre in the UHG (Type I 2.5 ± 0.6 Baseline vs. 2.5 ± 0.4 4 Weeks, $p = 0.95$; type II 2.9 ± 0.6 Baseline vs. 3.1 ± 0.4 4 Weeks, $p = 0.58$, Figure 5.8.2 panel C) nor in the DHG (type I 2.8 ± 0.1 Baseline vs. 3.1 ± 0.5 4 Weeks $p = 0.5$; type II 3.3 ± 0.2 Baseline vs. 3.3 ± 0.4 4 weeks, $p = 1$, Figure 5.8.2 panel D). There were no differences between the groups at baseline for type I ($p = 0.45$) and type II ($p = 0.14$) nor after 4 weeks of training for type I ($p = 0.17$) and type II ($p = 0.34$).

Myonuclear domain

The myonuclear domain determined by the CSA per myonuclei was consistent with no myonuclei increase in the UHG and remain unchanged, but also remained unchanged in the DHG. The MND was significantly larger in type II fibres in the UHG at baseline but not at the 4 week time point (type I 2228 ± 647 μm^2 vs. type II 2807 ± 697 μm^2 Baseline; type I 2517 ± 453 μm^2 vs. type II 2710 ± 582 4 Weeks, $p = 0.01$, Figure 5.8.2 panel F). There was no significant difference in MND size of neither type I ($p = 0.46$) nor type II ($p = 0.8$) fibres in the UHG in response to 4 weeks of HIIT. The MND was significantly larger in type II fibres in the DHG compared to type I fibres at baseline and 4 weeks. (type I 2272 ± 372 μm^2 vs. type II 2508 ± 389 μm^2 Baseline; type I 2820 ± 502 μm^2 vs. type II 3362 ± 224 μm^2 4 weeks, $p < 0.05$). There was no increase in MND size with 4 weeks of HIIT in type I fibres ($p > 0.05$). Although not statistically significant, there was an increase in the MND in the type II fibres ($p = 0.06$) with 4 weeks of training (Figure 5.8.2 panel F). There were no differences between in MND between the groups at any of the time points.

Exercise training induced changes to the muscle fibre morphology are summarized in a table in Appendix F.

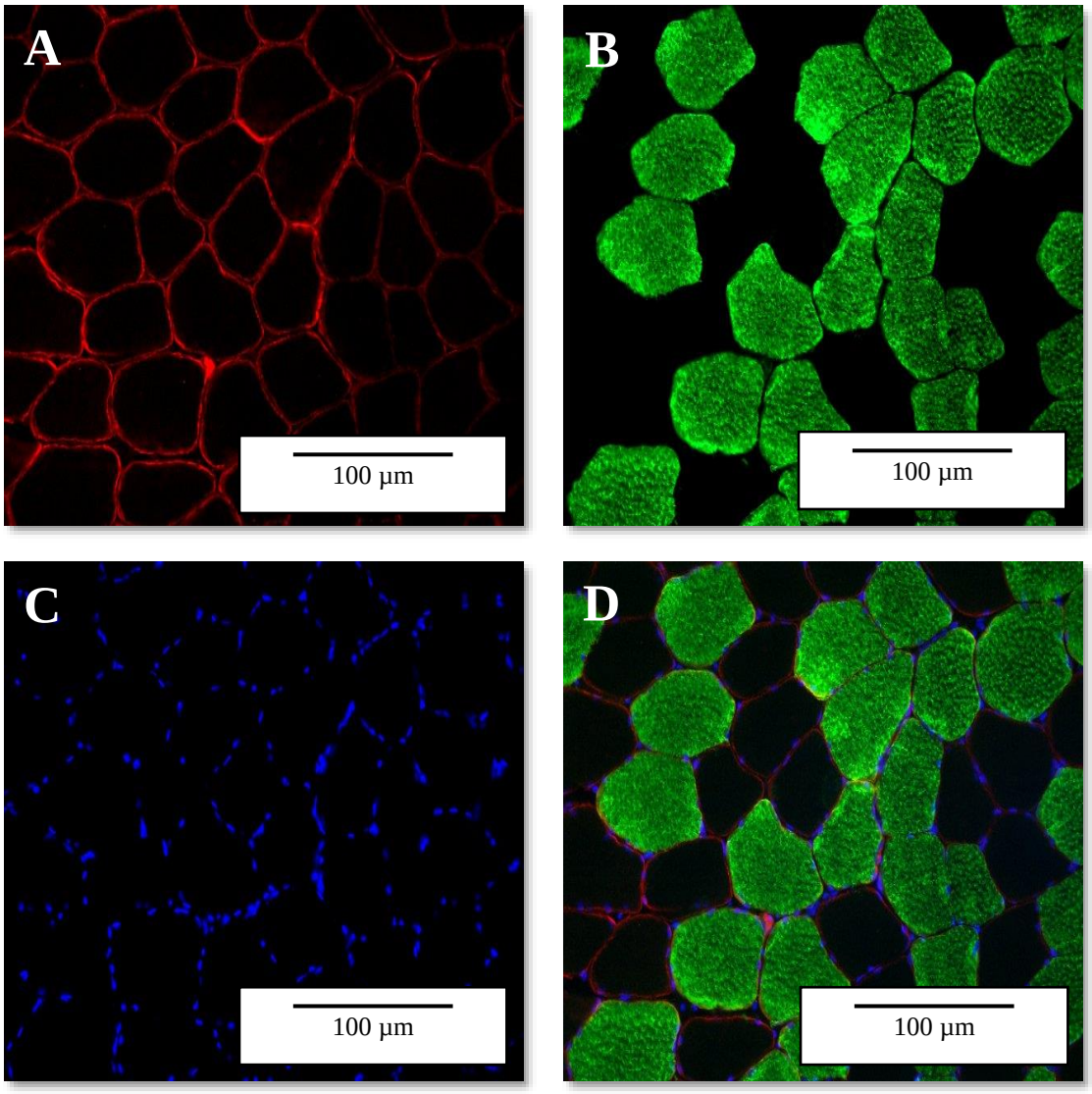


Figure 5.8.1 Muscle Cross Sectional Area and Fibre Type

Representative single view channels of A: Muscle fibre borders stained with dystrophin in red, B: Muscle fibre isoforms stained with MHCII in green, C: Nuclei stained with Hoechst in blue and D: Merged. Magnification 200X. Scale bar 100µm.

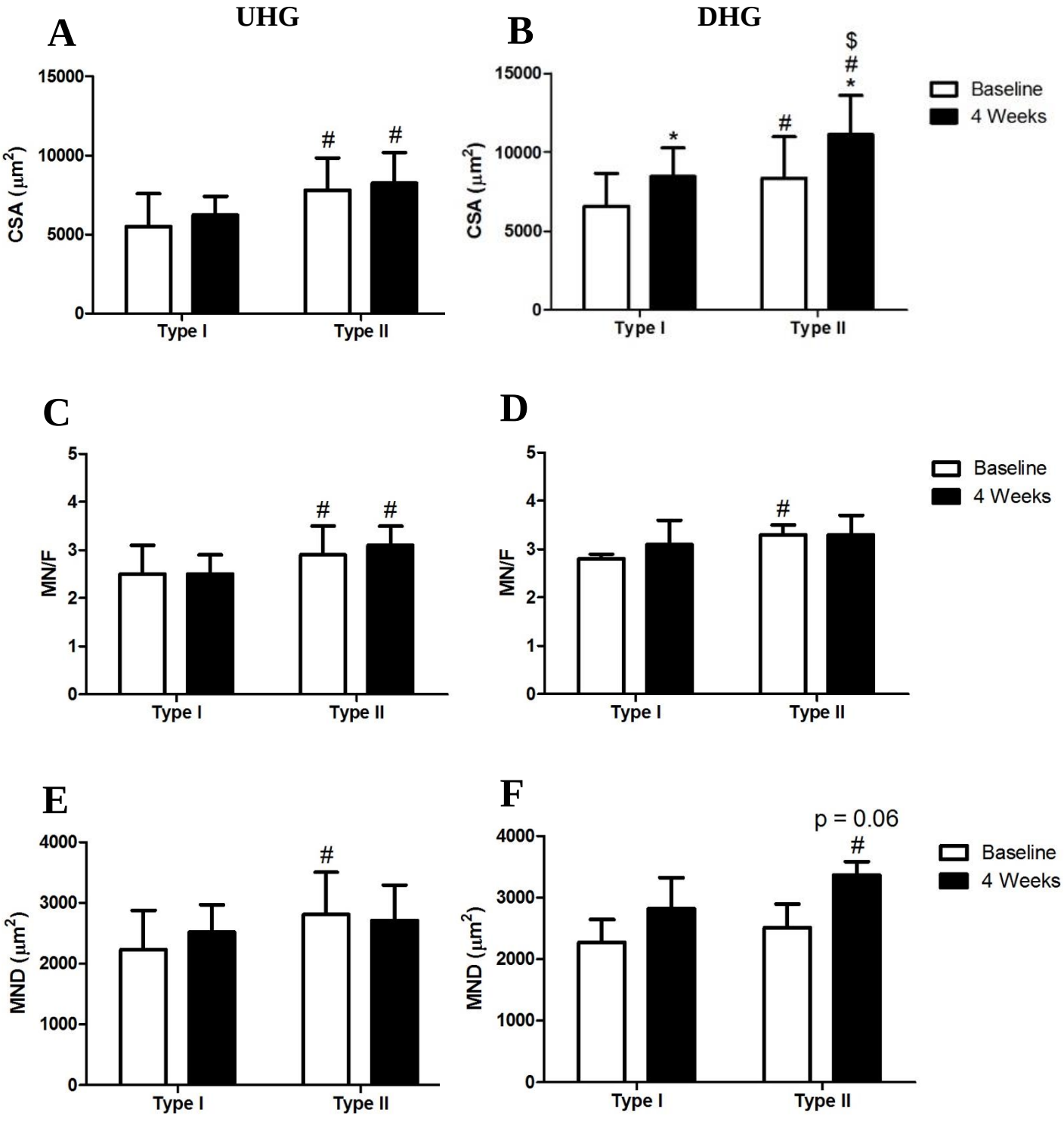


Figure 5.8.2 Changes within the muscle fibre in response to 4 weeks of HIIT

Muscle morphological characteristics of fibres of both type I and type II in response to 4 weeks of HIIT at the time points Baseline (white) and 4 Weeks (black). Panels A, C and E for UHG participants and Panels B, D and F for DHG participants. Abbreviations: y-axis explained. Data are expressed as mean ± SD. # Significance between fibre types, $p < 0.05$. * Significance between time points, $p < 0.05$. $ See text for difference between groups, $p < 0.05$. Trend of $p= 0.06$, not indicated by a symbol, refers to Type II baseline vs. 4 weeks.

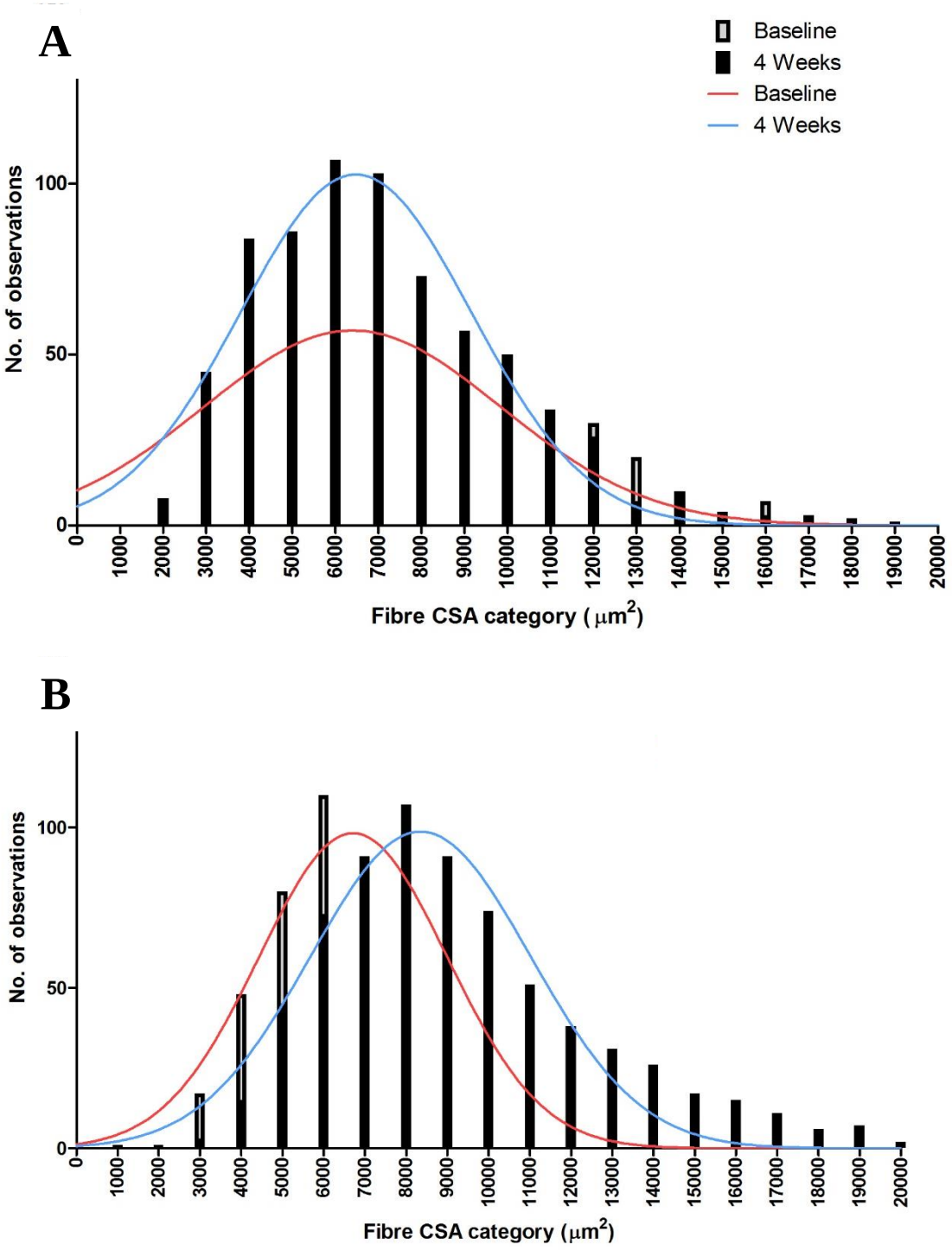


Figure 5.8.3 Frequency distribution of muscle fibre cross sectional area with 4 weeks of HIIT

A: Frequency distribution of the CSA of all measured fibres from all participants in the UHG (n=5) at the time point Baseline in grey and 4 Weeks in black. A frequency distribution curve is fitted for Baseline in red and 4 weeks in blue.

B: Frequency distribution of the CSA of all measured fibres from all participants in the DHG (n=6) at the time point Baseline in grey and 4 Weeks in black. A frequency distribution curve is fitted for Baseline in red and 4 weeks in blue.

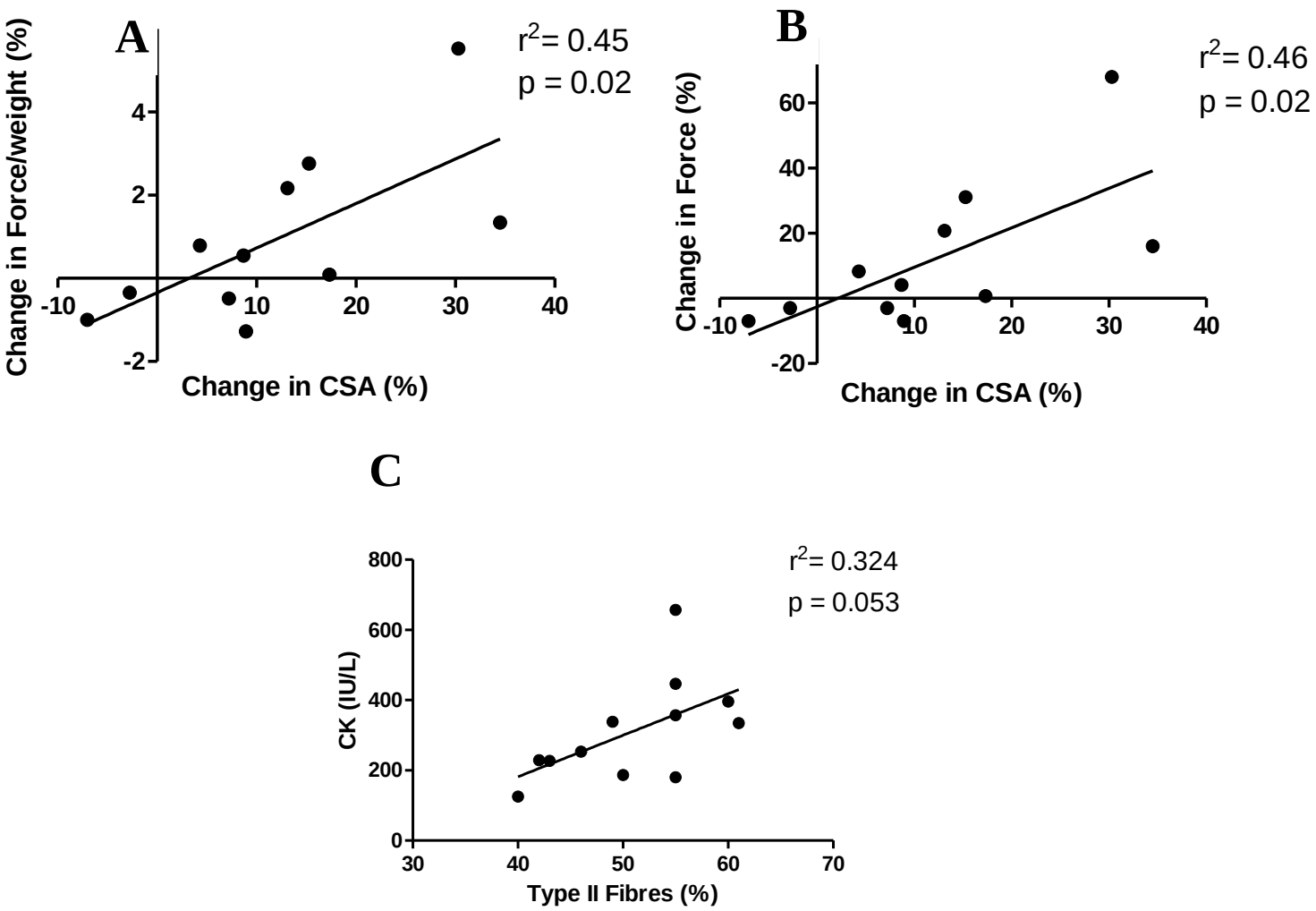


Figure 5.8.4 The correlation between the change in Cross sectional area and Force production in response to 4 weeks of HIIT and the correlation between creatine kinase activity and fibre type distribution.

A: Correlation between the change in CSA of all subjects (n=11) with 4 weeks of HIIT and the change in quadriceps maximal force expressed as force/weight in N/kg.

B: Correlation between the change in CSA of all subjects (n=11) with 4 weeks of HIIT and the change in quadriceps maximal force expressed as force in N.

Correlation between CK levels 6 hours aftr the first HIIT session, expressed in International Units per L, and the type II fibre type percentage of all participants (n=11).

$P < 0.05$ is considered significant.

5.9 Satellite Cells

Satellite cells were identified with immunofluorescence staining for MHCII to identify type II muscle fibres, Pax7 and Hoechst to identify the SCs and Wheat Germ Agglutinin to identify the muscle fibre border and correct location of SCs (Figure 5.9.1)

There was no increase in SC content in the UHG with from baseline compared to the acute time point and after 4 weeks of HIIT (0.10 ± 0.02 SC/Fibre Baseline vs. 0.10 ± 0.01 SC/Fibre Acute, $p = 0.53$, vs. 0.10 ± 0.01 SC/Fibre 4 weeks, $p = 0.83$) .The DHG had no significant increase in SC content following the first training session but there was a significant increase in SC content following training (0.10 ± 0.01 SC/Fibre Baseline vs. 0.09 ± 0.01 SC/Fibre Acute, $p = 0.6$, vs. 0.29 ± 0.02 SC/Fibre 4 Weeks, $p < 0.001$, figure 5.9.2 panel A). There was no significant difference in SC content between the UHG and the DHG at Baseline ($p = 0.74$) and Acute ($p = 0.42$). After 4 weeks of HIIT there was a significant difference in SC content between the groups (0.10 ± 0.02 UHG vs. 0.29 ± 0.02 DHG, $p < 0.001$).

There was no significant change from baseline in the fibre type specific SC content in the UHG 6 hours after the first session nor after 4 weeks of HIIT (type I 0.12 ± 0.03 SC/Fibre Baseline vs 0.11 ± 0.02 SC/Fibre Acute, $p = 0.27$, vs. 0.11 ± 0.01 SC/Fibre 4 Weeks, $p = 0.81$; type II 0.12 ± 0.03 SC/Fibre Baseline vs. 0.11 ± 0.03 SC/Fibre Acute, $p = 0.9$, vs. 0.10 ± 0.09 SC/Fibre 4 Weeks, $p = 0.62$, figure 5.9.2 panel B). There was no significant increase from baseline in fibre type specific SCs 6 hours after the first session in the DHG in type I fibres but there was a significant increase after 4 weeks of HIIT (0.10 ± 0.03 SC/Fibre Baseline vs. 0.10 ± 0.03 SC/Fibre Acute, $p = 0.65$, vs. 0.16 ± 0.07 SC/Fibre 4 Weeks, $p < 0.001$). Similarly, there was a significant increase in type II SC content in response to 4 weeks HIIT but not 6 hours after the first session (0.09 ± 0.01 SC/Fibre Baseline vs. 0.09 ± 0.01 SC/Fibre vs. 0.33 ± 0.04 SC/Fibre Weeks, $p < 0.001$, figure 5.9.2 panel C). There were no significant differences in fibre type specific SC content between the UHG and the DHG when comparing Baseline and Acute time points, but after 4 weeks of HIIT there was a significant difference in type II specific SC content between the groups (0.10 ± 0.91 SC/Type II Fibre UHG vs. 0.33 ± 0.02 SC/Type II Fibre DHG, $p < 0.001$).

Satellite cell pool size changes in response to 4 weeks of HIIT are summarized in a table in appendix F.

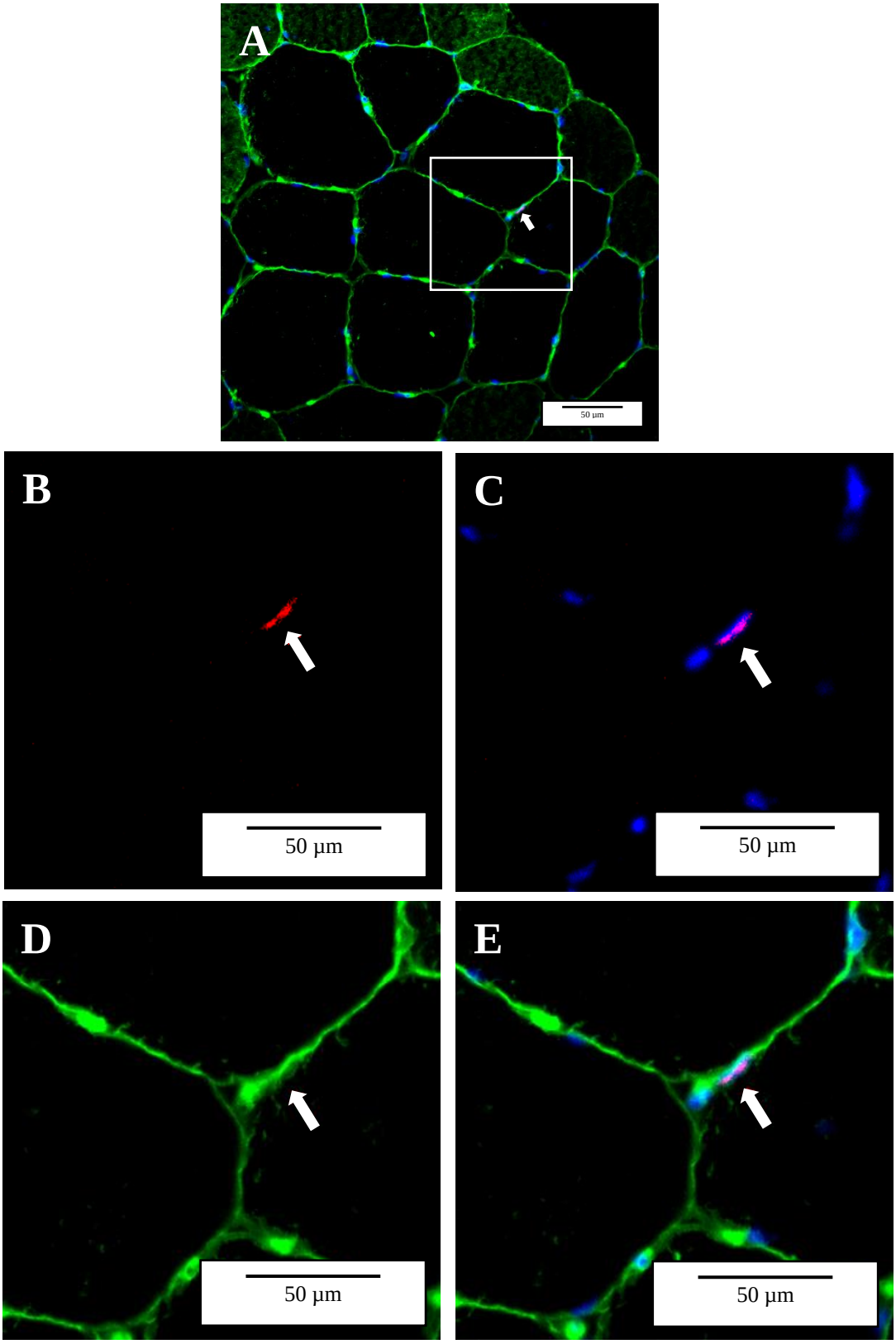


Figure 5.9.1 Satellite cell identification using Pax7

Representative single channel views of A: Muscle fibre types and nuclei. B: SC stained with Pax7 in red, white arrow. C: Nuclei stained with Hoechst in blue. D Muscle fibres and borders stained with MHCII and Wheat Germ Agglutinin respectively in green. Merged channel view E: showing the composition and location of muscle fibre types, SC and nuclei. Magnification 200X (A) and 400X (B-E). Scale bar 50µm

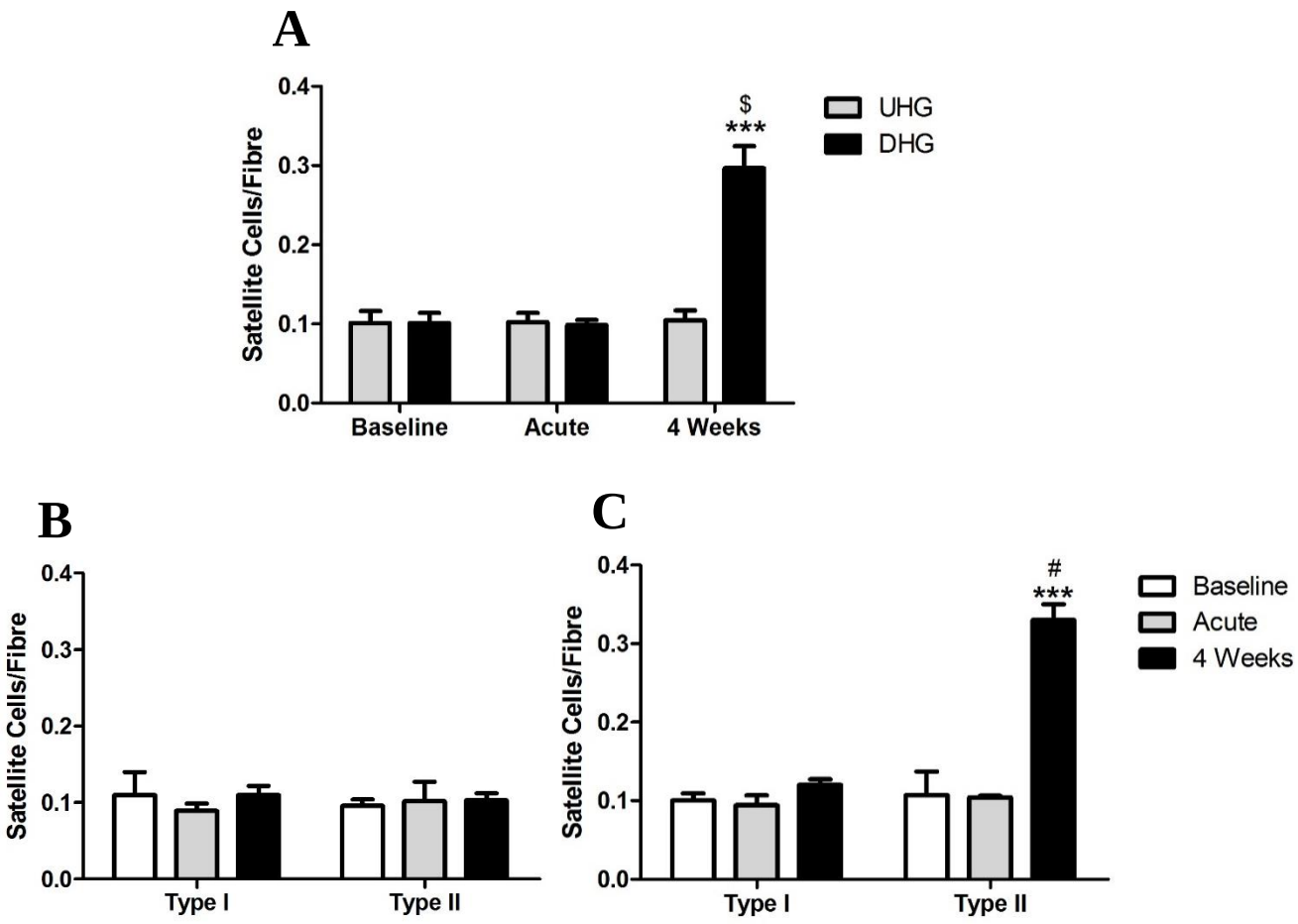


Figure 5.9.2 The change in the satellite cell pool size with 4 weeks of HIIT

A: The SC pool size change with 4 weeks of HIIT expressed as the number of $Pax7^+$ cells/fibre from all participants in the UHG (n=5) in grey, and the DHG (n=6) in black.

B: The fibre type specific SC pool size expressed as the number of $Pax7^+$ cells/ fibre from all participants in the UHG (n=5) in both type I and type II muscle fibres.

C: The fibre type specific SC pool size expressed as the number of $Pax7^+$ cells/ fibre from all participants in the DHG (n=6) in both type I and type II muscle fibres.

Data are expressed as Mean ± SD.

*** Significantly different from other time points ($p < 0.001$).

4.10 Myogenic Regulatory Factors

4.10.1 MyoD

There was no significant difference in MyoD protein content between the UHG and the DHG. The MyoD response in the UHG was unchanged from baseline at the acute time point ($p = 0.41$) and after 4 weeks HIIT ($p = 0.387$, figure 5.10.1). When assessing the individual MyoD response to training in the UHG, an n= 2 subjects responded with an increase in MyoD protein content at the Acute time point compared to Baseline, but at levels lower than that of the mean response of the DHG (Figure 5.10.2). There was a significant increase from baseline in MyoD after the first session ($p = 0.01$) which then returned to baseline levels with 4 weeks of training ($p < 0.001$, figure 5.10.1). There was no significant difference in MyoD protein between Baseline and 4 Weeks ($p = 0.56$). To further understand the change in MyoD protein content over time in the DHG it was shown that n=4 subjects responded to the 1st bout of DH running with an increased MyoD protein content at the Acute time point compared to Baseline, the levels returned to baseline levels at the 4 week time point (Figure 5.10.2).

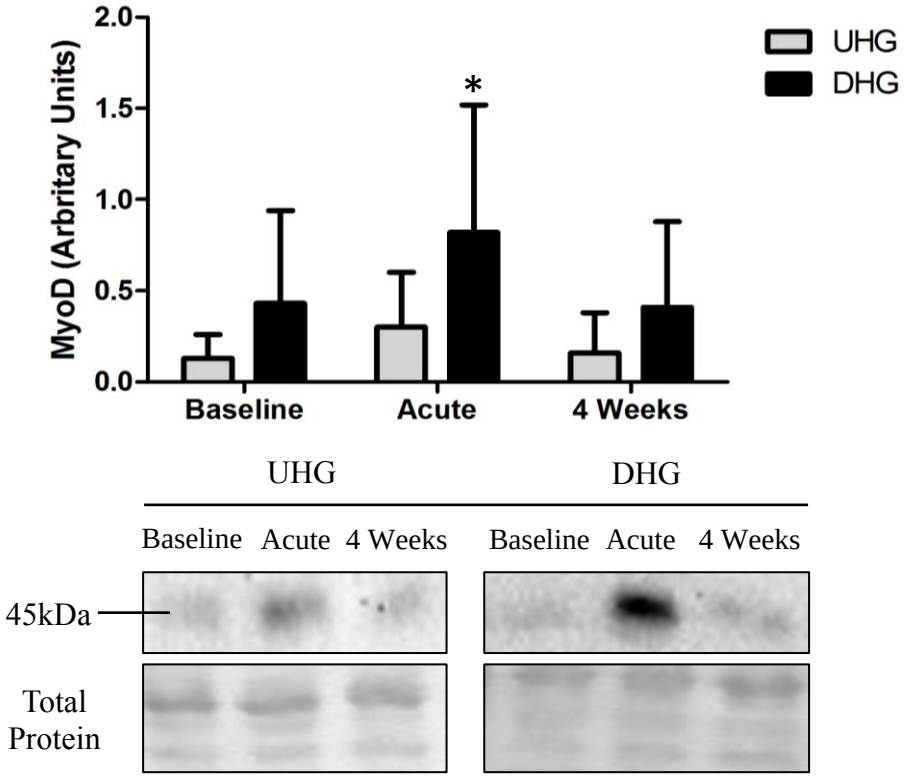


Figure 5.10.1 MyoD protein response to 4 weeks of HIIT

The change in MyoD protein content for all participants from the UHG (n=5) in grey and the DHG (n=6) in black. MyoD shows bands at approximately 45kDa. Protein was normalized to total protein. Data are expressed as Mean ± SD. * significant between time points $p < 0.05$

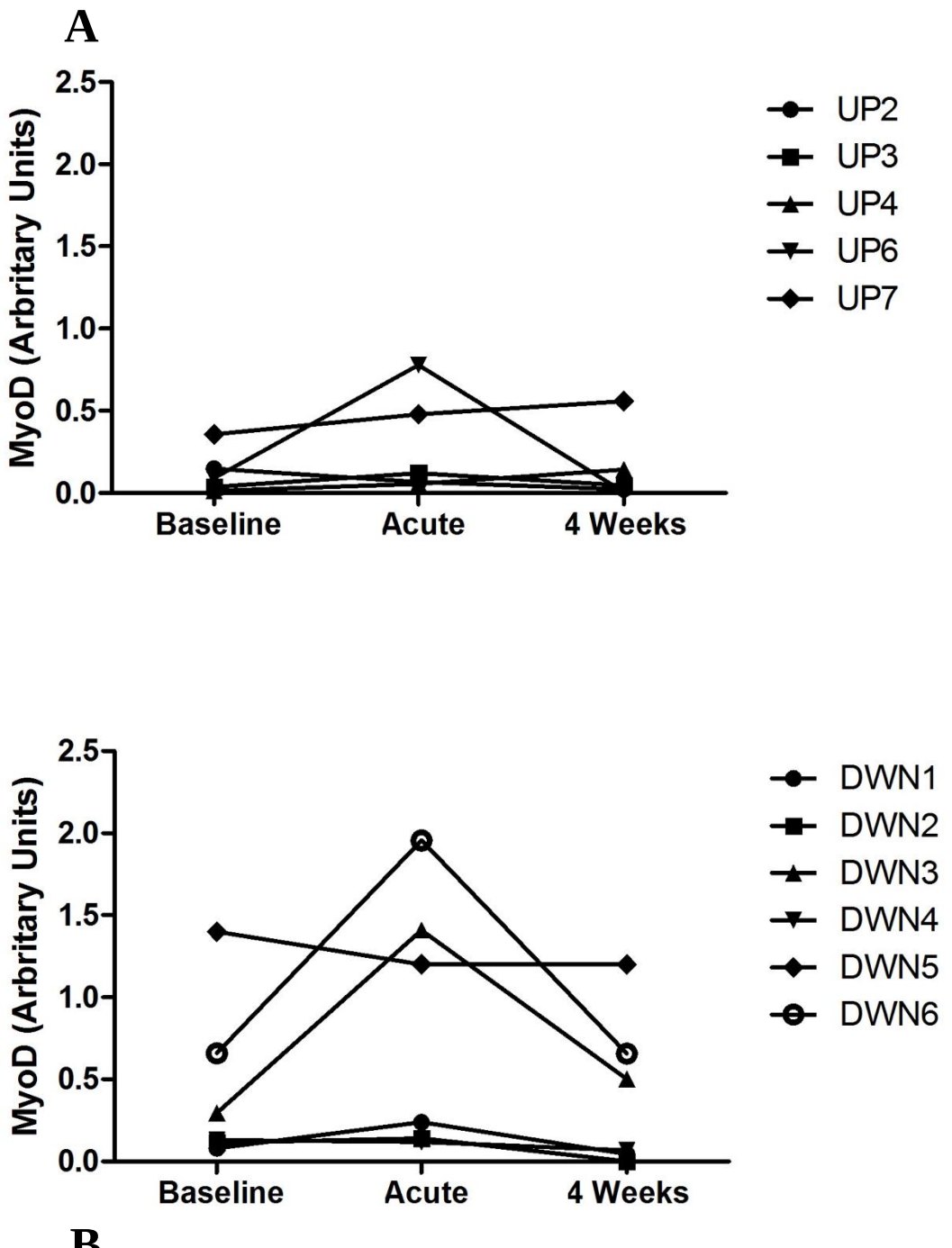


Figure 5.10.2 The individual MyoD protein response

The individual myoD protein response at the baseline, acute and the 4 week time points for all individuals in the UHG (n=5) in panel A and the DHG (n=6) panel B. Data are expressed as arbitrary units normalized to total protein.

4.10.2 Myogenin

There was no significant difference in myogenin protein content between the UHG and the DHG. There was no significant change from baseline in myogenin protein content in the UHG with 4 weeks of HIIT. (0.397 ± 0.655 AU Baseline vs. 0.270 ± 0.045 AU Acute, p = 0.64 vs. 0.152 ± 0.157 AU 4 Weeks, p = 0.13, figure 5.10.3). When assessing the individual myogenin response to 4 weeks of HIIT, none of the subjects responded with increased myogenin at either the Acute or the 4 week time points. One subject's baseline myogenin level was significantly higher than the other subjects, but this subjects myogenin levels follow similar levels at the Acute and 4 week time point (Figure 5.10.4). This subject was not a baseline outlier during any of the other muscular assessments. Similar to the UHG, the myogenin protein content in the DHG did not significantly change with 4 weeks of HIIT (0.549 ± 0.642 AU Baseline vs. 0.776 ± 0.988 AU Acute, p = 0.65 vs. 0.171 ± 0.231 AU 4 Weeks, p = 0.08, figure 5.10.3). When assessing the individual myogenin response to training only n=1 subject responded at the Acute time point and n=0 responded at the 4 week time point (Figure 5.10.4) One subject's baseline myogenin level was significantly higher at baseline, increasing at the Acute time point and decreasing to a level lower than baseline after 4 weeks of HIIT.

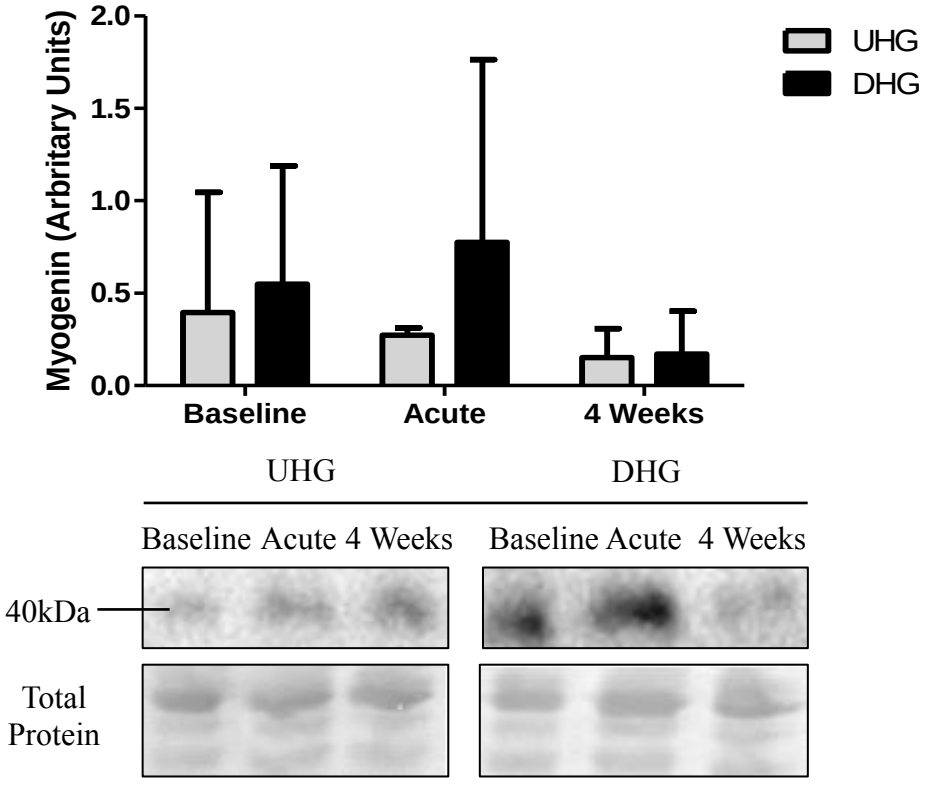


Figure 5.10.3 Myogenin protein response to 4 weeks of HIIT

The change in Myogenin protein content for all participants from the UHG (n=5) in grey and the DHG (n=6) in black. Myogenin shows bands at approximately 40kDa. Protein was normalized to total protein. Data are expressed as Mean ± SD.

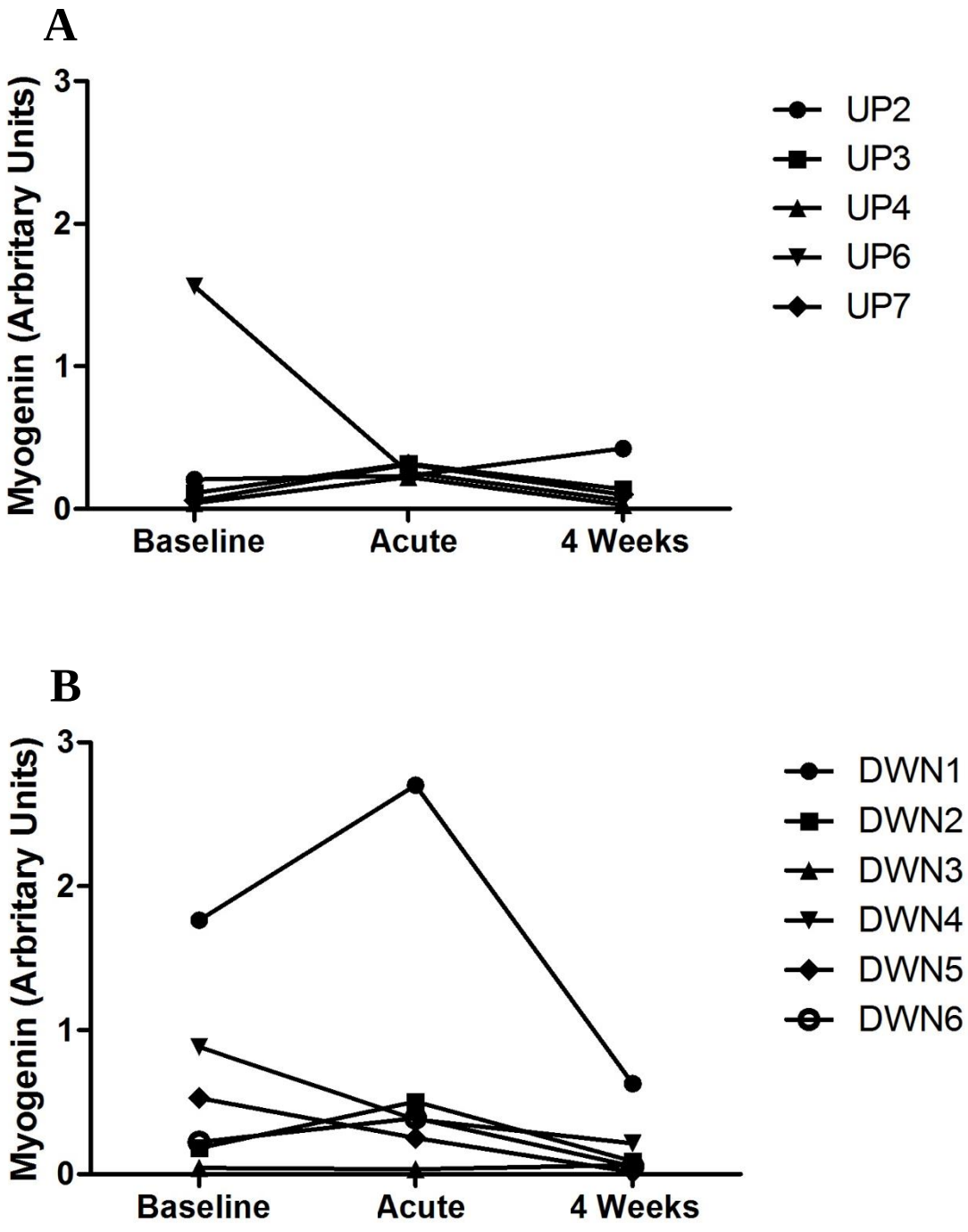


Figure 5.10.4 The individual Myogenin protein response
The individual myogenin protein response at the baseline, acute and the 4 week time points for all individuals in the UHG (n=5) in panel A and the DHG (n=6) panel B. Data are expressed as arbitrary units normalized to total protein.

5.11 Muscle Capillaries

Capillaries were identified by means of immunofluorescence staining of MHCII to identify type II muscle fibres, CD31 to identify capillaries, Wheat Germ Agglutinin to identify the muscle fibre border and Hoechst to identify the nuclei (Figure 5.11.2).

There was a significant increase in the capillary to fibre ratio in the UHG in response to 4 weeks of HIIT (1.76 ± 0.18 C:F Baseline vs. 2.55 ± 0.20 C:F 4 Weeks, $p < 0.001$, Figure 5.11.1 panel A) but not in the DHG (1.61 ± 0.27 C:F Baseline vs. 1.7 ± 0.16 C:F 4 Weeks, $p = 0.62$). There was no significant difference between the UHG and the DHG at baseline ($p = 0.67$) but after 4 weeks of HIIT there was a significant difference between the groups ($p < 0.001$).

Similarly, there was a significant increase in capillary density in response to 4 weeks HIIT in the UHG (249 ± 39 capillaries/mm^2 Baseline vs. 304 ± 57 capillaries/mm^2 4 Weeks, $p = 0.04$, figure 5.11.1 panel B) but not in the DHG (217 ± 12 capillaries/mm^2 Baseline vs. 194 ± 44 capillaries/mm^2 4 weeks, p 0.84). There was no significant difference between the UHG and the DHG at baseline but after 4 weeks of HIIT there was a significant difference between the groups ($p = 0.01$). Muscle capillary changes in response to 4 weeks of HIIT are summarized in a table in appendix F.

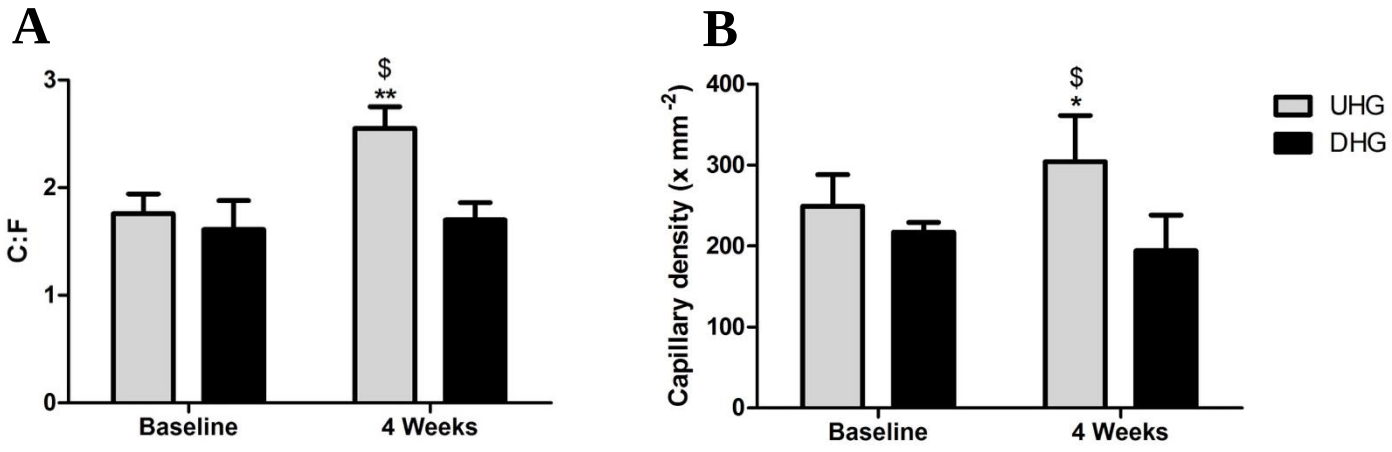


Figure 5.11.1 The change in the capillary per fibre ratio with 4 Weeks of HIIT
The C:F ratio of all participants in the UHG (n=5) in grey, and the DHG (n=6) in black at Baseline, and after 4 weeks of training.
Data are expressed as Mean ± SD. ** significantly different from other time points ($p < 0.01$).

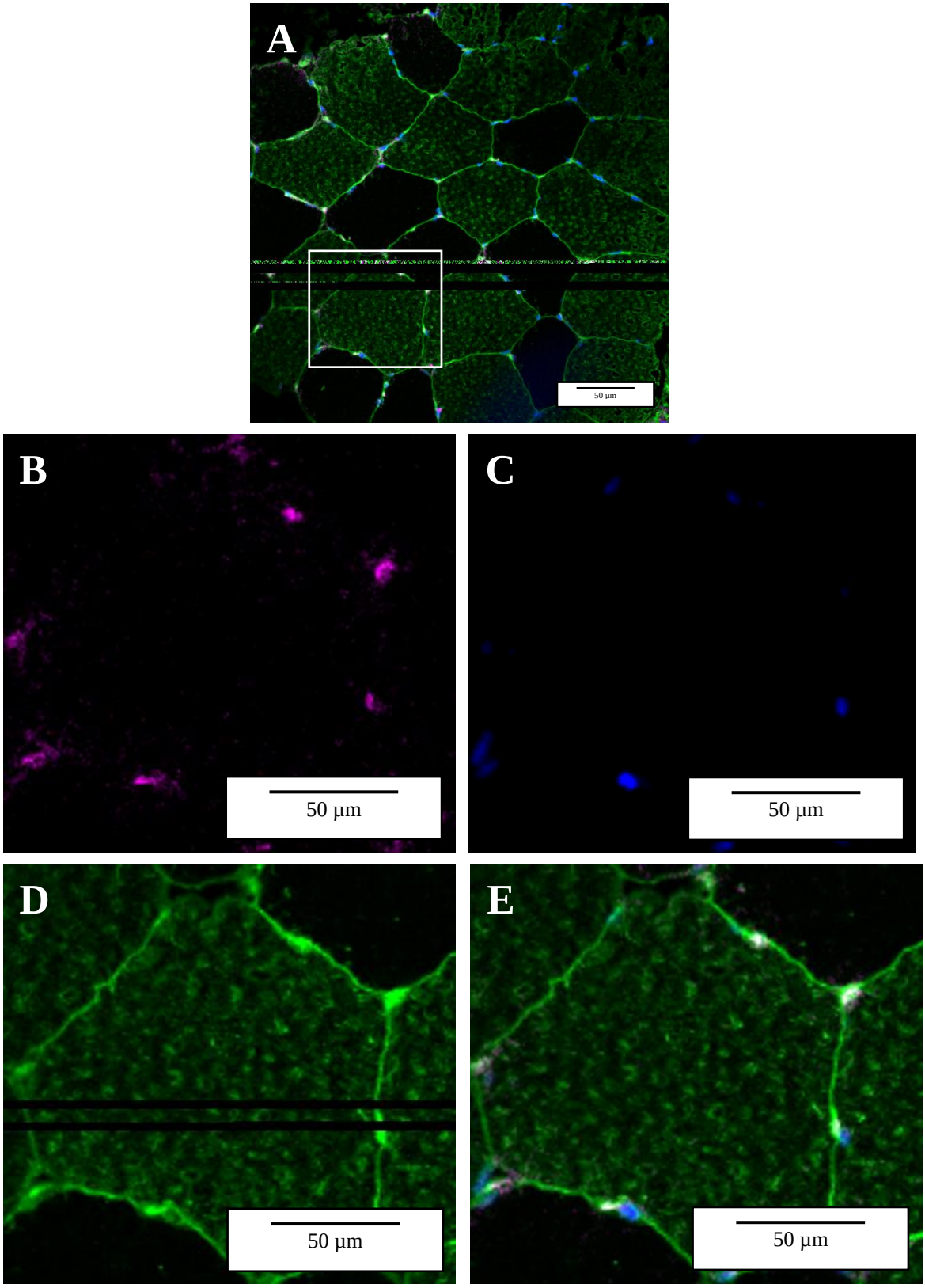


Figure 5.11.2 Muscle capillaries

Representative single channel view of A: Muscle fibre types and nuclei. B: Capillaries stained with CD31 in purple. C: Nuclei stained with Hoechst D: Fibre type and borders stained with MHCII and Wheat Germ Agglutinin respectively. Merged channel E: showing the location of muscle capillaries within the muscle fibres. Magnification 200X (A) and 400X (B-D). Scale bar 50 µm.

5.12 Relationship between SC and capillaries

The spatial relationship between SCs and capillaries was done by means of immunofluorescence staining whereby MHCII was used to identify fibre types, WGA to identify the fibre borders, Pax7 to identify SCs, CD31 to identify capillaries and Hoechst to identify the nuclei. The distance between a SC and its nearest capillary was done to measure the relationship between the two (figure 5.12).

There was no significant difference in the distance between SC and its nearest capillary in the UHG over the 4 weeks of HIIT. When assessing the fibre type specific SCs and the distance to the closest capillary there was no significance in type I nor type II fibre specific SCs and its nearest capillary over the 4 weeks of HIIT, nor a difference between the fibre types. Similar to the UHG there was no significant difference in the distance between a SC and its nearest capillary over the 4 weeks of HIIT in the DHG. The fibre type specific SC and the distance to its nearest capillary also showed no significant difference between the fibre types and no change over 4 weeks of HIIT. There was no significant difference between the UHG and the DHG at any of the time points. The distance between a SC and its nearest capillary is summarized in table 5.12.

Table 5.12 The spatial relationship between satellite cells and capillaries

		UPHILL			DOWNHILL		
	Fibre Type	**Baseline**	**Acute**	**4 Weeks**	**Baseline**	**Acute**	**4 Weeks**
Distance to closest capillary (μm)	I & II	23.4 ± 3.9	20.5 ± 5.1	22.2 ± 2.3	19.3 ± 5.0	22.5 ± 1.1	20.7 ± 3.5
	I	22.4 ± 2.8	19.6 ± 5.5	22.6 ± 2.3	20 ± 5.3	23.3 ± 2.8	19.9 ± 3.2
	II	20.7 ± 5.0	21.1 ± 5.2	22.0 ± 2.9	16.4 ± 3.8	23.4 ± 1.4	21.1 ± 4.1

Data expressed as Mean ± SD

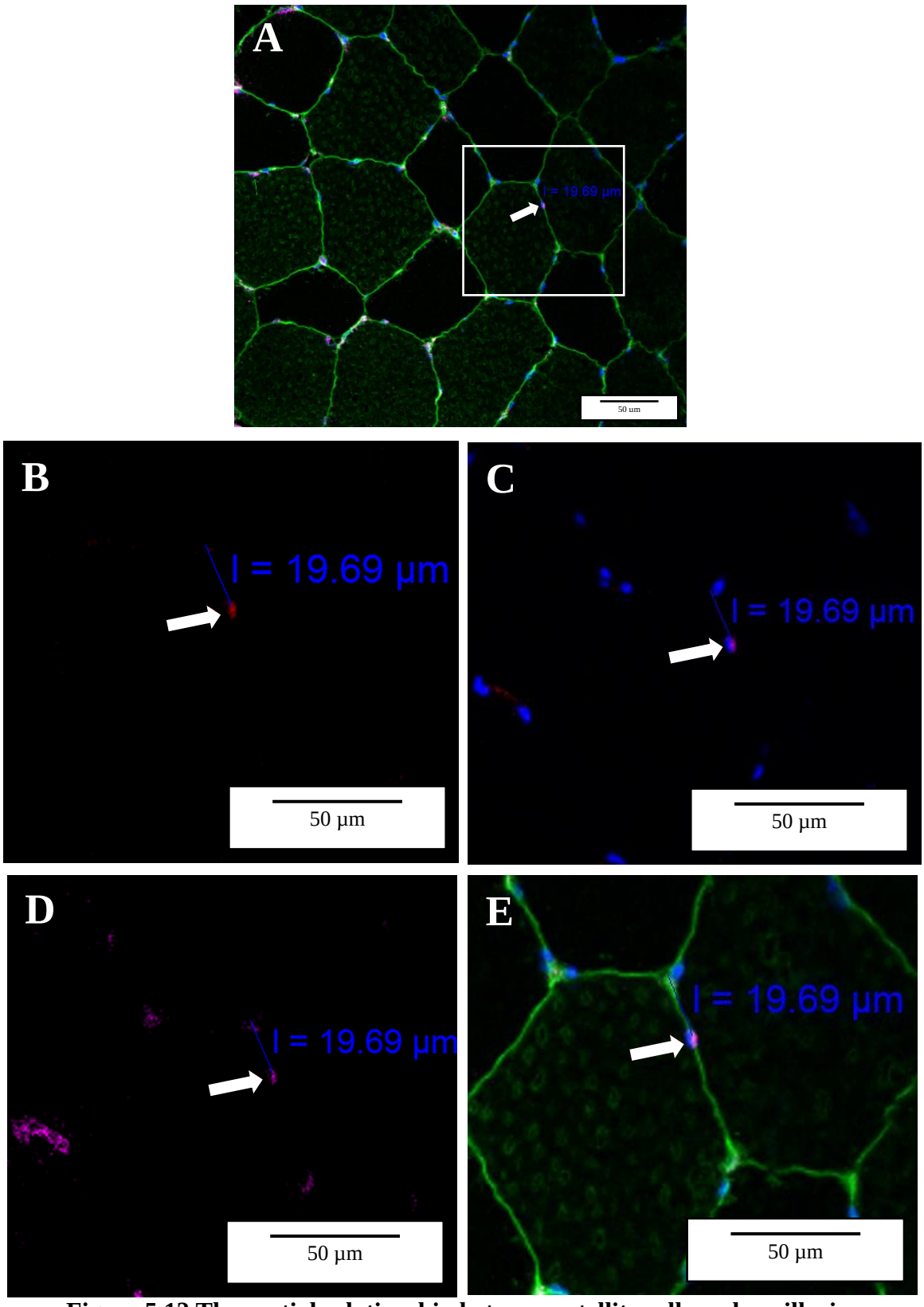


Figure 5.12 The spatial relationship between satellite cells and capillaries

Representative merged image of the distance between a SC and its nearest capillary A. Multi-channel, B zoomed in SC stained with Pax7 in red C: Nuclei stained with Hoechst in blue D: Capillaries stained with CD31 in purple and E: muscle fibre borders stained with WGA and type stained with MHCII in green, merged image. White arrow indicates a SC. Blue line indicates the distance between the SC and its nearest capillary. Magnification 200X (A) and 400X (B-E). Scale bar 50 µm.

Chapter 6: Discussion

The research design of this thesis was set up to compare two training intervention protocols. The discussion section will first address how successfully these protocols were implemented. The one protocol (DHR) was expected to induce some muscle damage and the next part of the discussion will assess if this occurred, as well as whether or not the UHG exhibited any evidence of muscle damage.

One aspect of muscle adaptation to training is morphological change. The main finding for this category of possible change was that the muscle fibre CSA changed, although only in in the DHG, whilst the number of myonuclei per fibre and the myonuclear domain size did not change in either group. After placing these results in the context of the literature, they will be interpreted in the light of the findings related to satellite cells and myonuclei. Thereafter, the limited available evidence collected in this thesis on recapitulation of the myogenic regulatory pathway will be discussed.

Another aspect of muscle adaptation to training is metabolic adaptation, which also includes some related morphological changes. In this thesis the main morphological variables related to metabolic adaptation was quantification of capillarisation. The UHG had an increased muscle capillarisation after training, whilst the DHG did not. The major differences in adaptation between the two HIIT intervention groups was an increased CSA and SC pool size in the DHG, which remained unchanged in the UHG and an increased muscle capillarisation in the UHG which remained unchanged in the DHG.

6.1 Training intervention

The DHG were able to complete every interval in every training session, whilst the UHG could not, but with training the number of intervals that could be completed per session increased. This was in conjunction with a higher RPE reported by uphill subjects compared to downhill subjects. Previous research has reported that uphill running at the same velocity as downhill running causes a change in the pH of the muscle to a more acidic pH compared to level surface and downhill running (236), blood lactate levels are found to be higher in uphill running compared to downhill running (237, 238, 239). The combination of a lower pH and

higher blood lactate could be indications of metabolic overload and thus transient muscle fatigue could be the result, explaining why the RPE was higher in the UHG and the subjects fatigued before completing the 6 intervals in a session.

A higher RPE has also been reported in uphill runners compared to downhill runners at the end of an either uphill or downhill repeated sprint protocol (239). For the research presented in this thesis, difference in running speed between the groups was expected and the groups were matched by heart rate, which is a matching method that has been reported in literature (237); although it has also been reported that the HR response to uphill running is greater than that of downhill running (239).

The external training load of the subjects remained constant over the 4 weeks of HIIT. Additional training load was due to the study intervention, which suggests that muscular and performance adaptations are due to the 4 weeks of HIIT and no external or new training. Of importance, is that the HIIT did not replace any of the other training, as is sometimes done for HIIT studies (240).

6.2 Indirect markers of muscle damage

Serum CK activities vary among individuals according to multiple factors. Ethnic black men have elevated CK levels compared to Caucasian men at rest and without intervention that may elevate this parameter (55). Here, one study participant who had an elevated baseline CK was of the coloured population of the Western Cape (South Africa). Whilst there have not been any systematic studies on this population's resting, unprovoked CK activity, it could be suggested that ethnicity is the reason of the elevated baseline CK. Two individuals with mildly elevated baseline CK activities (UHG n=1 and DHG n=1) were the only two individuals who recreationally took part in resistance training. This could be explained by the fact that individuals who frequently train have elevated CK levels (53). Nonetheless, their CK activities were still within the range of 335 – 362 international units/L which is well below the level indicating muscle damage. The fibre type distribution of an individual may also contribute to CK activity variability, as individuals with a greater type II percentage have a higher CK response (241). Here, the percentage of type II fibres did not significantly

correlate with the CK response after the first training session, but was considered to be a statistical trend (p = 0.056). Variation was observed in the fibre type distribution amongst the subjects, potentially adding to the variation in the CK response.

Due to natural variation in baseline CK activity the data were analysed as the percentage change from baseline. The DHG had 2 CK non-responders, adding to the variation in CK response and thus the DHG responders group (n=.4) was compared to the UHG. When the two subjects were excluded from the CK analysis there was elevated serum CK activity and PMP 6 hours after the first training session in the DHG but not the UHG. The DHG responders (n=4) had an increase in CK activity after the first training session of every week, but not to the same extent as after the first training session. The UHG did not have a significant CK activity response after any training session. The PMP was lower 6 hours after the 10^{th} session compared to 6 hours after the 1^{st} session, calculated for the subjects in the DHG, whilst there was again no increase in PMP in the UHG. Literature supports these findings as elevated CK and DOMS was found in subjects after downhill running but not after uphill running (242, 243). The blunted CK response and PMP as the 4 weeks of training commenced in the DHG could be due to the onset of muscular adaptation and the repeated bout effect, which is supported by a decreased CK response on subsequent bouts of eccentric exercise (54).

6.3 Central nuclei

Central nuclei within the muscle fibre are an indication of fibre regeneration from damage or, depending on the size of the fibre, new fibre formation (244). In states of skeletal muscle pathology there is an increase in central nuclei in an attempt to generate new muscle contractile, cytoskeletal and other elements to compensate for the damaged muscle undergoing destruction. There was an increase in the number of central nuclei per fibre in the DHG after the first bout of eccentric running, compared to baseline levels and the UHG. The number of central nuclei after the last exercise session was significantly lower than after first session. After muscle damage central nuclei have been shown to be one of the final stages of myogenesis in new fibre formation, as the newly differentiated myoblast fuses into the damaged fibre and localizes in the centre of the fibre (nuclear centration) (244). It is unlikely

that new fibre formation occurred so soon after the first bout of eccentric running; there was no increase in myogenin at the same time point, suggesting that terminal differentiation of SCs had not yet taken place. The increased central nuclei could then be as a result of the eccentric induced muscle damage. A greater number of central nuclei have been noticed in biopsy samples from body builders, who undergo extensive resistance training, compared to that of sedentary individuals (245). Within the DHG, there were statistically significantly more centrally located nuclei in type II fibres compared to type I fibres.

Eccentric exercise preferentially damages type II fibres (40), which would explain in the observed increased number of central nuclei in those fibres. The UHG did not have centrally located nuclei; uphill running places less mechanical stress on the muscle than downhill running and as a result less muscle damage occurs, which could explain why there was an increased number of central nuclei in the DHG compared to the UHG after training. The number of centrally located nuclei decreased after the 4 weeks of HIIT in the DHG, back to baseline levels. One can conclude that with repetitive bouts of eccentric exercise, the muscle had adapted to the mechanical stress placed on it and therefore less muscle damage occurred in the last HIIT session prior to the biopsy, thus requiring less repair and thus resulting in fewer central nuclei.

6.4 Fibre type distribution

The individual variation in fibre type distribution before training was high, although not significantly different between the two groups. There was no change in fibre type distribution with training in either the UHG or the DHG. This may firstly be due to the high natural variation. Secondly when assessing muscle physiology by looking at multiple *vastus lateralis* muscle biopsies from the same individual, there may be a natural degree of variation of fibre type without any intervention (246). This second explanation does not exclude the first explanation, but rather simply adds to the variability that makes it difficult to determine significant fibre type change. Thirdly, the number of HIIT sessions and the duration over which these took place may have been too short for fibre type distribution to change. A 15-week cycle HIIT protocol has been shown to change the proportion of muscle fibre types, with a larger percentage of type I and a lower percentage of type IIx fibres whilst type IIa

fibres remained unchanged (247). This protocol was almost 4 times the duration of the HIIT protocol in this study. There may be fibre type changes if the current HIIT protocols (either UH or DH) were carried out for longer than 4 weeks. Other adaptations that precede a shift in fibre type such as an increase in mitochondrial density and oxidative enzyme activity, which increases with training (87, 88), were not assessed in the current study. It would have been beneficial if this study identified the difference between type IIa and IIx to assess if there was a shift in type II fibre iso-form towards a more oxidative or glycolytic fibre distribution.

6.5 Muscle fibre morphology and isometric force after 4 weeks of either UH or DH HIIT

There was a significant increase in CSA with 4 weeks of HIIT in the DHG but not the UHG, in both type I and type II fibres. Repetitive eccentric exercise activates growth signalling pathways in the muscle itself, resulting in the activation of genes in the myonuclei for protein synthesis and inhibition of genes for protein degradation (248). While eccentric exercise causes muscle damage predominantly to type II fibres, type I fibres are still damaged and the current results of centralised nuclei in both fibre types suggest muscle damage occurred in both fibre types and hypertrophy was not fibre type-specific hypertrophy. It may depend on the exercise type, whether or not there is a fibre type-specific hypertrophic response. A 15 week cycling HIIT protocol was completed by healthy sedentary men and woman. There was an increase the CSA in type I and type IIa fibres, but not type IIx fibres (247). This type of training unlike downhill running does not induce hypertrophy via muscle damage, but most likely mechanical strain due to recruitment and power output required for pedalling. Cycling HIIT could utilise the oxidative fibres more than the glycolytic fibres, causing these fibres to undergo hypertrophy, regardless of whether the fibres were type I oxidative or type IIa oxidative. The UHG in the current study did not undergo hypertrophy in any fibre type. It could be concluded that the 4 weeks of HIIT was insufficient stimulus to cause non-damaging contraction-induced type I hypertrophy, unlike other studies which induced hypertrophy that had a longer training phase (189). Molecular mechanisms for the hypertrophy associated with eccentric contractions have been investigated by other researchers. In a study assessing the myostatin activity after an eccentric, concentric or isometric training, myostatin mRNA levels decrease after exercise, regardless of contraction mode but the largest decrease was post eccentric training, promoting greater protein synthesis (249).

The consequence of increased CSA would include the capacity for a more forceful contraction. All subjects in the DHG had an increase in muscle fibre CSA and increased maximal isometric force, (6%-90% increase); in terms of DH running this would allow the muscle to withstand the load of the eccentric contraction more efficiently, resulting in less muscle damage. A major finding of the current study was that only the DHG improved their maximal isometric force with 4 weeks of HIIT. Because only one of the HIIT protocols provided this adaptation, analysis of the change in CSA of the muscle fibres with training did not significantly correlate with the change in muscle strength when assessing all of the study participants together. There was a significant correlation between the change in fibre CSA and isometric force with 4 weeks of HIIT, since those subjects with most increase in CSA above 20% also had the greatest increases in force.

6.6 SC content and myonuclei adaptation and their relationship to CSA

There was no increase in the SC pool for the UHG post training, but there was a 3-fold increase in the DHG indicating significant activation and proliferation and maintenance of the expanded pool size over the weeks. Fibre type-specific SC analysis revealed an increase in the type II specific SCs in the DHG post training. Eccentric exercise causes muscle damage, where the SCs respond to regenerate the injured muscle. Downhill running is considered as an eccentrically biased exercise on the *quadriceps* muscles, of which the *vastus lateralis* which was biopsied is one. The CK activity responses over time indicated that the repeated eccentric exercise continued to induce muscle damage over the 4 weeks of training and although biopsies were not taken at all these time points, the maintenance of the expanded pool size suggests activation and proliferation of the SCs continued over time. A greater inflammatory response was noted after a single bout of downhill running compared to uphill running (242). Cytokines and growth factors are known to stimulate SC activity and nudge them along the myogenic path (166). IL-6, for example, is released from contracting muscle with increased levels after downhill running (155). IL-6 activates the SC by activating the JAK/STAT pathway (159). Therefore a greater inflammatory response following downhill running may have contributed to the increased SC proliferation, although inflammatory parameters were not measured.

Following a single bout of eccentric exercise, the number of SCs per fibre increased at 12, 24 and 72 hours post exercise, with peak numbers at 72 hours post exercise (168). Resistance training for 16 weeks increased the SC pool in both type I and type II fibres (114). The increased SC pool size post training in the current study could potentially be considered not only a response to each DH HIIT bout, but a training adaptation to this regular stimulus. Such an adaption may allow for an increased myogenic response to subsequent eccentric bouts of exercise. Typically, during the myogenesis of fetal development, neonatal and subsequent growth, proliferated SCs fuse with each other or existing fibres to enlarge these either longitudinally or in cross-section (250). In the adult longitudinal growth has halted, it isn't clear if SCs that have proliferated in response to a stimulus will usually fuse or not.

The number of myonuclei per fibre was bigger in type II fibres compared to type I fibres in both the UHG and the DHG. This finding was expected as it is proposed that the MND is consistent in resting muscle. A bigger fibre area in type II fibres and the increased number of myonuclei in those fibres results in a similar MND between the two diverse fibre types. As stated above, there was an increase in the SC pool size with 4 weeks of HIIT in the DHG. However, no increase in the number of myonuclei per fibre was observed, suggesting that the SCs did not fuse into the adult muscle fibres. An alternative explanation is that the eccentric exercise may have caused a small degree of myonuclear apoptosis, while the regeneration response of the muscle including the SC activation and proliferation may well have culminated in a limited amount of SC fusion into the regenerating muscle fibres to replace lost myonuclei, but not adding significantly to nuclear accretion. Nuclear accretion is usually associated with hypertrophy, thus myonuclear number remains consistent. It has been relatively well established in literature that hypertrophy due to resistance training is associated with an increased number of myonuclei due to SC fusion into the myofiber (251, 183, 184). The current study reports hypertrophy due to a downhill running protocol, with no increase in myonuclear number per fibre but an increased SC pool size outside the fibres.

In a point-counterpoint debate (252), O'Connor and Pavlath provide insight into criterion used to assess the contribution of SCs to hypertrophy. These authors suggest that studies must provide evidence on necessary time points of the hypertrophic phase, an increased CSA after the hypertrophic intervention, distinguishing between myonuclei and other nuclei and cause

and effect of SC activity rather than correlations. They suggest the MND to be the mediator of SC accretion, while McCarthy supplied a counterpoint argument. This author points out that various publications of MND report results for this parameter that are too variable and without more consistent findings on MND size, conclusions on its role in SC fusion cannot be made (252). Methods to assess the MND are predominantly imaging of stained muscle fibres in the cross section. Few studies have assessed the MND in the longitudinal section. Perhaps the manner in which the MND is being assessed is not specific enough to answer all related research questions.

Another point that is not in the counterpoint debate, is that the majority of the literature on SC pool size increase and myonuclear addition in human volunteers is due to a resistance training protocol. These protocols vary in exercise movement type, intensity and load, providing a range of very variable stimuli even when the pre-protocol training status of the volunteers is not factored in. It then becomes difficult to compare muscular adaptations between protocols let alone different exercise modalities. The exercise intervention may result in a different magnitude of certain autocrine, paracrine, hormonal, cell signalling and inflammatory responses. Also, volunteers recruited with widely differing inclusion and exclusion criteria may not be comparable for these mechanistic responses that could influence SC pool or MND or both.

6.7 Myogenic response to HIIT

Counting of SC provides evidence of whether or not activation and proliferation occurred. To determine progress along the myogenic path, analysis of one or more of the myogenic regulatory factors could be useful. There was an increase in MyoD levels in the DHG 6 hours after the first bout of exercise compared to their baseline while MyoD of the UHG was unchanged at the same time point. Following the 4 weeks of HIIT, MyoD levels were not significantly different from baseline in either the UHG or the DHG. Myogenin protein levels remained unchanged from baseline at both 6 hours after the first bout of exercise and after 4 weeks of HIIT. These data suggest that SC did not progress significantly down the myogenic path. There were 2 subjects with elevated baseline myogenin levels (n=1 from each group). Potentially these individuals may have performed an activity which induced a transient

myogenin response which was detected at the baseline time point of this study. Neither of these individuals had elevated serum CK levels, an increase of central nuclei nor an increased SC pool, suggesting no baseline muscle damage. Comparing the current results with other studies revealed that there is a slight lack in consistency in literature when assessing MRF responses to exercise. It has been shown that there is no change in mRNA of MyoD or Myogenin 8 hours after resistance exercise (253). In contrast, a different study reported that resistance training resulted in an increase in P21 and MyoD mRNA levels 3 hours after resistance exercise, but not Myogenin mRNA. That latter is not consistent between studies, since a different study found all three of Myf5, MyoD and Myogenin mRNA is increased 3 hours after resistance training (169). For protein levels to be changed, mRNA must first be translated and a different time course of response might be expected. MyoD protein levels have been reported to be increased 24 hours after of resistance exercise (254). Therefore, different methods of assessing the MRF responses must be taken into account. In the studies just mentioned, whether the parameters were mRNA or protein, they were determined from small portions of the muscle biopsies. Another approach is to determine if SC themselves express MRFs. The number of MyoD positive SCs has been shown to be increased at 12, 24, 48, and 72 hours following a bout of resistance training and in this particular, study the authors also determined that there was an increase in myoD and myogenin protein levels in extracts of the biopsy samples taken 12-48 hours after exercise (168).

Literature describing the myogenic responses to running are even less conclusive. Level surface treadmill running for 45 minutes did not change mRNA levels for MyoD and Myogenin in biopsies of the *vastus lateralis* taken 4 hours after exercise, but an upregulation was seen in MRF4 mRNA. Biopsies from the *soleus* had an increase in mRNA for both MyoD and MRF4, whilst Myogenin remained unaltered (255). These subjects had a mean VO_{2max} of 77 $ml.kg^{-1}.min^{-1}$, suggesting that they were well trained athletes. Thus muscle adaptation to training may have already taken place so that one bout of level surface running may have been an insufficient stimulus to induce a robust and consistent MRF response. However, this study demonstrated the potential for muscle specific adaptation to running training. Running training enhances the IL-6 and IGF-1 in circulation following exercise (256). IL-6 is known to play a role in SC proliferation (158), and IGF-1 is known to have a dual effect on proliferation and differentiation (142).

In the current investigation, myoD and myogenin protein levels were determined at 6 hours after the first HIIT session. Results suggest an increase in MyoD levels was more prominent in some individuals than others, and no increase in myogenin protein was seen. The running training may have resulted in an increase of IL-6, HGF and IGF-1 isoform responsible for SC activation and proliferation but inhibiting differentiation. Literature suggests that the 6 hour time point may be too early to measure peak protein expression, and hence an absence of change at the early time point, does not mean that a sample taken later would also reveal no response. The addition of PCR analysis of the MRF mRNA in the muscle biopsies could have reinforced conclusions made about SC activity after an acute bout of HIIT and training. Measuring SC activation, with either the use of PCNA or Myf-5 co-staining could also have provided more evidence to understand the SC activity. Western blot analysis was performed with Myf-5, but issues with the antibody and time constraints terminated the experiment.

In the current investigation, a 6 hour biopsy was also taken after the last HIIT session. After the 4 weeks of HIIT, the muscle may have adapted to the mechanical stress of downhill running, and this would explain why no MyoD or Myogenin mRNA was upregulated suggesting that the SC pool size measured at this time was not a new acute response. The inhibition of differentiation could have returned the SCs to quiescence without reduction in pool size. These findings combined with the increased SC pool size but lack of nuclear accretion in the DHG, could suggest that the training was sufficient stimulus to induce a SC response where the SC proliferates and starts to differentiate sufficiently to supply signals to regenerate damaged muscle, but without fusion. Increasing the training load by either increasing the speed, making the gradient steeper or increasing training frequency will be unaccustomed to the muscle and might induce a further SC response in the DHG.

As mentioned before, there was no increase in the SC pool for the UHG post training. The lengthy discussion above is relevant only to the DHG, but the following sections are relevant only to the UHG. Since the two protocols resulted in divergent adaptative responses.

6.8 Muscle capillary response

There was an increase in C:F and capillary density in response to 4 weeks of HIIT in the UHG but not in the DHG. In this study, the number of capillaries per muscle fibre ranged from 1.5, at baseline, to 2.9, after 4 weeks of HIIT. These values are consistent with the literature (206). Extreme endurance runners have C:F as high as 5.8- 8.5 (257). Capillary density in this study is therefore at values less than that in the literature for aerobically well-trained individuals (223).

Consistent aerobic training increases capillarisation in skeletal muscle. There is a lot of literature showing this with the use of cycling training protocols at various intensities and durations (226, 223). For example, Gavin et al. (2007) explain that after 8 weeks of cycle training at 65% of the VO_2max, the mean capillary to fibre ratio of young sedentary males increased from 1.42 at baseline to 1.80. Aerobic running training too increases capillarisation in skeletal muscle (206, 222). The same is not noticed, however, in level surface sprint HIIT for 8 weeks (224). The runners recruited for the study by Gliemann et al. (2015) had similar VO_{2max} and 5 km time trial times and similar performance improvements to that of this study, without adaptation to the muscle vasculature. Indicating that sprint training may enhance performance through other muscular adaptation.

Uphill running has an increased metabolic demand compared to both level surface and downhill running (86). The increased metabolic demand with the 4 week UH HIIT protocol may be a potent stimulator for angiogenesis. The increase in capillarisation allows for more efficient oxygen and nutrient transfer to the muscle and waste removal from the muscle. Increased capillarisation results in an improved performance during high intensity exercise bouts to exhaustion (258), which could explain the improvement in VO_{2max} after the 4 weeks of training in the UHG and no change in the DHG. Downhill running in rats causes capillary destabilization due to mechanical stress on the muscle and capillaries (259), although this might not be a potent enough response to promote angiogenesis. Other models with eccentric contractions such as resistance training have resulted in an increase in capillarisation (225, 114), but resistance training has a large concentric component which could influence muscular adaptation. Downhill running has less of a concentric contraction component.

6.9 Spatial relationship between the SC and capillary

The mean distance between the SC and its nearest capillary did not change from baseline for either type I or type II specific SCs in response to training in either of the groups. What was not measured was the relative frequency of SCs that were situated nearer and further away. It has been shown that activated SCs are situated closer to capillaries than quiescent SCs (203). Activated SCs were not identified in this study, which could have provided a clearer explanation on the relationship between SCs and capillaries after UH or DH HIIT.

6. 10 Performance improvements with 4 weeks of HIIT

The UHG had an improvement in VO_{2max} and PTS with 4 weeks of HIIT, whereas the DHG had no change in this performance variable. On the other hand, the DHG improved muscle force production where the UHG did not. Both the UHG and the DHG had a 3% improvement in 5 km time trial performance.

Similar results have been published in the literature which reports that uphill running HIIT for 6 weeks increased VO_{2max} and the duration that the velocity at VO_{2max} could be maintained in well trained runners (260). Ferely et al. (2013) also investigated different training velocities for uphill running and performance improvements and concluded that training at the velocity associated with 75-85 % of VO_{2max} on an incline (6 weeks) resulted in the ability of the runners to reach the peak treadmill speed achieved from level surface running, when running on an incline (260). The UHG increased their PTS from a mean of 17.5 km/h to 18.5 km/h. In terms of the incremental exercise test used in this study, subjects from the UHG were able to run at velocities associated with their baseline VO_2max for a longer period of time. . Improving the ability to run at a velocity just under the VO_2max may be a powerful indication of racing performance (261). The metabolic stress of uphill running HIIT resulted in adaptation potentially enhancing the buffering capacity of the muscle to maintain pH more efficiently as has been shown for HIIT in cyclists by Weston et al (1997) and more efficient lactate clearance, as has been shown for cyclists an a continuous training program (262). There was an increase in muscle capillarisation in the UHG, an adaptation associated with greater VO_{2max} performance and a higher anaerobic threshold (263). It has been suggested that using the VO_{2max} and the velocity at VO_{2max} correlates with 8 km running

performance (264). Although the statistical calculations were not done in this study due to lower subject numbers, it could be said that this same relationship exists with performance over 5 km, in the UHG but not the DHG suggesting that another factor improving the DHG 5 km time trial performance was present.

A more forceful contraction allows a runner to cover a greater distance with each stride (265). The spring constant during the eccentric phase of the stretch-shortening cycle increases as running velocity increases (266). Downhill running strengthens the eccentric contraction, and thus could result in a more efficient eccentric phase in the stretch-shortening cycle resulting in a more forceful contraction during running. Additionally a significant correlation exists between 5 km time trial performance and EMG at the ground contact phase of running (267). Although EMG was not tested in the current study, there is a force-EMG relationship at the ground contact phase of running (268). Previously it has been shown that the introduction of a plyometric training intervention as part of the training regimen of endurance athletes trained the eccentric component of the stretch-shortening cycle and improved their running performance by 3.9% (269). It could be inferred that the improvement in muscle force production in the DHG may have played a role in their improved running performance, suggesting an improvement in running performance independent of VO_{2max} and other related parameters.

In summary, it is very well established that running training will improve performance (270, 271, 272). The question is more about the details of the training protocol in terms of type, duration, intensity and frequency. Both moderate intensity exercise and HIIT result in similar performance improvements (95). Here it has been described how different modes of HIIT induce a similar performance response over a 5 km time trial.

Chapter 7: Conclusion, limitations and future recommendations

Uphill HIIT has an increased metabolic stress on the muscle which resulted in a greater oxidative capacity of skeletal muscle indicated by an increase in VO_2max and an increased muscle perfusion. The increased perfusion allows more efficient O_2 and nutrient delivery to the muscle and waste removal from the muscle. Downhill HIIT places an increased mechanical stress on the muscle which resulted in eccentric exercise induced muscle damage and a SC response to regenerate damage fibres. There is an increased CSA to withstand the force placed on the muscle by eccentric contractions. There is an increase in SC pool, most likely to induce an accelerated myogenic response to subsequent unaccustomed eccentric bouts.

This proves the hypothesis that 4 weeks of HIIT will induce muscular adaptation in a contraction specific manner, although from measurements presented in this study, it seems only the downhill group had a SC response.

This study has revealed that 4 weeks of uphill or downhill HIIT results in physiological adaptation by different mechanisms, one involving muscle perfusion and oxygen utilization and another by enhanced SC and a more forceful contraction. The mechanisms of adaptation are training specific yet they both result in a similar improvement in 5km race performance. This gives athlete coaches a better understanding of the muscular adaptation associated with uphill and downhill running. In order to optimize performance an athlete will either have to increase speed, for example, which can be achieved by an increased muscle force output or an improved metabolic system. Coaches and athletes can identify which of the two might need improvement and train at the necessary gradient.

The application of this research exists beyond the realm of athletics and performance. Understanding the role of the SC allows researchers and medical practitioners to combat myopathies and muscle wasting with disease. This research adds to the body of literature of the SC, adding an element of exercise specificity and intensity in understanding the role of

the SC and hypertrophy. This is the first study, to my knowledge, which compares the muscular adaptation due to uphill or downhill running HIIT.

7.1 Limitations and future recommendations:

There is natural variation between human participants, especially in a study which sets out to assess the response of an individual to an intervention. A sample size of only n=5 and n=6 in the UHG and DHG respectively is too little to eliminate inter-subject variation. The lack of controls is a limitation. A flat surface group would have added a more interesting comparison to assess if gradient running provides runners with better adaptation to training. This study did not have any control groups to compare to. A non-exercising control group would strengthen the data. This study did not compare HIIT to traditional moderate intensity continuous aerobic training. Future studies should include the addition of a moderate intensity control group, matched to the HIIT with duration of running time. This could allow for comparison to address the matter if HIIT is a superior training modality compared to the more traditional continuous training. Additionally it would be beneficial to introduce a combined group, running both uphill and downhill to assess if both muscular adaptations assessed in this study could be achieved at once, and what impact that would have on 5km time trial time. What needs to be thought about critically when adding a group of this nature would be to determine the training volume. If the volume remains the same as in this study, then the intervention would have half the uphill and downhill training volume of this study. Will that be sufficient volume to induce adaptation? If the uphill and downhill training remains the same as this study the volume will be doubled, can comparisons be made?

In assessing the muscular adaptations due to training, there was no method to determine the difference between type IIa and IIx muscle fibres. The identification of fibre type iso-forms could provide a clearer understanding to potential fibre type shifts, if any, and fibre type adaptation to training.

Myogenesis could not entirely be assessed as there was only a baseline and 6 hour post exercise time point. These time points may have “missed” the peak levels of myogenic regulatory factors. Future studies should include the addition of time points at 12, 24 and 72

hours after exercise in order to fully monitor myogenesis, although it might be a challenge to recruit human volunteers for this number of muscle biopsies. Additionally, qPCR data of myoD and myogenin would strengthen the analysis of myogenesis and help understand the SC response to training.

When assessing the relationship between SCs and capillaries there was no indication of the activation status of the SC. Identifying what stage of myogenesis each SC is and then measuring the distance to its closest capillary might give a clearer indication of the relationship between the SCs and the microvasculature.

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Appendices

Appendix A: Ethical approval

UNIVERSITEIT
STELLENBOSCH
UNIVERSITY

Approved

New Application

Health Research Ethics Committee (HREC)

15/01/2018

Project Reference #: 1781

HREC Reference #: S17/10/240

Title: Comparison of two high intensity exercise training protocols on satellite cell dynamics and microRNA release

Dear Mr Cameron Sugden,

The **New Application** received on 30/10/2017 was reviewed by members of the **Health Research Ethics Committee 2 (HREC 2)** via **expedited** review procedures on 15/01/2018 and was approved.

Please note the following information about your approved research protocol:

Protocol Approval Period: **This project has approval for 12 months from the date of this letter.**

Please remember to use your **Project Reference Number [1781]** on any documents or correspondence with the HREC concerning your research protocol.

Please note that this decision will be ratified at the next HREC full committee meeting. HREC reserves the right to suspend the approval and to request changes or clarifications from applicants. The coordinator will notify the applicant (and if applicable, the supervisor) of the changes or suspension within 1 day of receiving the notice of suspension from HREC. HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review

Please note you can submit your progress report through the online ethics application process, available at: https://apply.ethics.sun.ac.za and the application should be submitted to the Committee before the year has expired. Please see Forms and Instructions on our HREC website for guidance on how to submit a progress report.

The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility, permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Please consult the Western Cape Government website for access to the online Health Research Approval Process, see: https://www.westerncape.gov.za/general-publication/health-research-approval-process. Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.

For standard HREC forms and instructions, please visit: Forms and Instructions on our HREC website (www.sun.ac.za/healthresearchethics)

If you have any questions or need further assistance, please contact the HREC office at 021 938 9677.

Yours sincerely,

Francis Masiye,

Appendix B: Recruitment Flyer

WHAT'S BETTER FOR MY 5KM TIME TRIAL, MUSCULAR STRENGTH AND AEROBIC FITNESS?

UPHILL OR DOWNHILL HIT TRAINING?

Stellenbosch University's Department of Physiological Sciences is recruiting male participants for a research study. This study aims to find out how different types of high intensity training protocols can affect muscle adaptation.

To take part, you must be:

- A healthy male
- Between the age of 18 – 29
- Not using pain or anti-inflammatory medication, cream or patches
- Moderately trained: Able to run 5km between 20 and 25 minutes
- Non-smoker

What will your participation involve?

- You will be invited to attend an initial information session at the Department of Physiological Sciences, where you can make an informed decision on whether you wish to take part in this study.

- **Visit 1** (±30 mins): Explanation and informed consent
- **Visit 2** (±1 hour): Familiarisation exercise session
- **Visit 3** (±1 hour): 5km Time trial
- **Visit 4** (±20 mins): 1 x muscle biopsy and 1 x blood draw
- **Visit 5** (±1 hour): 1 x Performance testing
- **Visit 6** (±1 hour): HIT session (±1 hour) 1x muscle biopsy and 2 x blood draws
- **Visits 7- 16** (±1 hour): High intensity training sessions – x10
- **Visit 17** (±1 hour): 5km Time trial
- **Visit 18** (±1 hour): 1 x Performance testing
- **Visit 19** (±1 hour): HIT session 1 x muscle biopsy and 2 x blood draws
- **Visits 20- 27** (±1 hour): High intensity training session – x8
- **Visit 28** (±20 mins): 1 x muscle biopsy and 1x blood draw
- **Visit 29** (±1 hour): 5km Time trial
- **Visit 30** (±1 hour): 1 x Performance testing
- **Visit 31** (±1 hour): HIT session 1 x muscle biopsy and 2 x blood draws

Remuneration of R2000 will be given to each volunteer upon the completion of the study, their time commitment and adherence to the protocol and aims.

For further details please contact Mr. Cameron Sugden on 17854687@sun.ac.za or 0609971252

Appendix C: Snap Freezing in OCT Protocol

Tissues should be kept moist and cool until snap freezing procedure is started

1. Gently blot excess liquid off of tissue.

2. Fill an appropriate container with liquid nitrogen.

3. Immerse metal cup filled 3/4 full with isopentane into the liquid nitrogen. The levels of these two solutions should be the same for even freezing of your specimen.

4. The isopentane will look opaque white and will have a rim of frozen isopentane when it is chilled enough to snap freeze a specimen (*keep adding liquid nit to your container to keep the level of the two liquids equal while you are waiting for the isopentane to chill).

5. Place tissue into a cut in half pipette tip (which will act as the mould) filled with Tissue Tek. Add more Tissue Tek to cover the sample

6. While holding the pipette tip mould between a pair of long handled forceps, immerse it down into the metal cup containing the chilled isopentane. Do not let go of the mould into the isopentane. Let it freeze for approximately 20-50 seconds (depending on size/thickness of tissue).

7. After freezing in the isopentane, quickly place your frozen tissue into the liquid nitrogen until you can store in a -80° freezer.

Appendix D: Immunofluorescence staining

Immunofluorescence staining Protocol

1. Thaw slides for 10 minutes at room temperature
2. Fix slides in 4% Paraformaldehyde for 10 minutes
3. Was sections with 1X PBS-T for 5 minutes 3 times
4. Block using 5% Donkey Serum and 1% Bovine Serum Albumin in 1X PBS for 90 minutes
5. Incubate sections in Primary Antibody at 4 degrees Celsius over night
6. Wash sections with 1X PBS-T for 5 minutes 3 times
7. All following steps must be performed in a dark room
8. Incubate sections in Secondary Antibody at room temperature for 1 hour
9. Wash sections with 1X PBS-T for 5 minutes 3 times
10. Add the following primary antibody. Perform steps 5-9 until the tissue have been labled with all primary and accompanying secondary antibody
11. Stain with Hoechst 1:1000 for 10 minutes at room temperature
12. Wash sections with 1X PBS-T for 5 minutes 3 times
13. Dry sections and mount with a coverslip using Dako fluorescent mounting media
14. Store at -20 degrees Celcius for short term storage

Making up Solutions

4% Paraformaldehyde

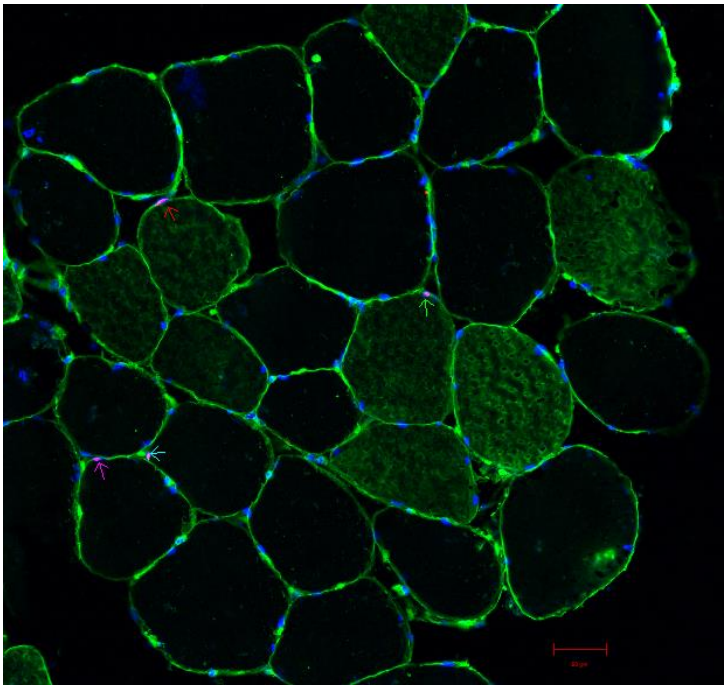
- To make up 1L add the following:
- 800mL 1X PBS to a glass beaker suitable for heating
- Add a magnetic mixer and place on a heated stirrer, heating the solution to roughly 50 degrees Celsius and a mild stirring speed.
- Add 40g PFA powder
- Adjust to pH to 7.4
- Add 1XPBS to 1L

1XPBS-T

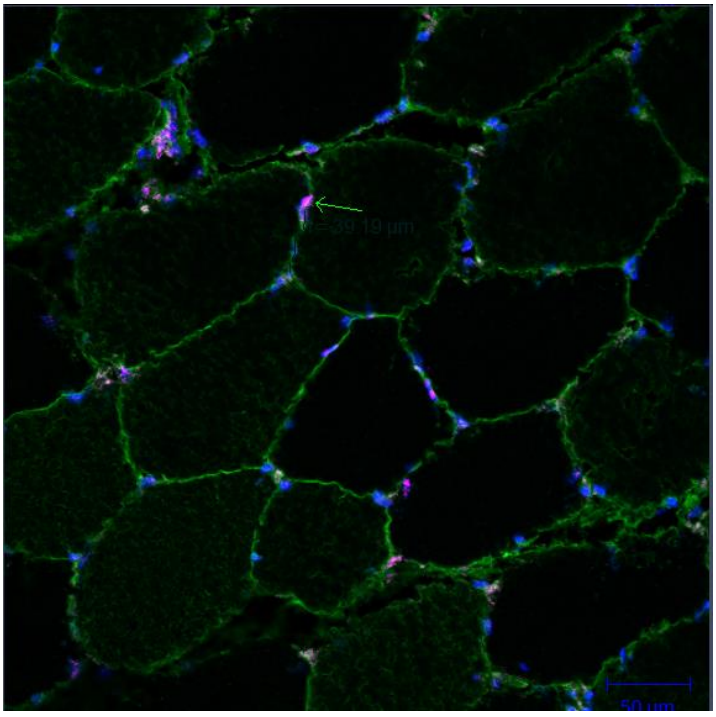
- To make up 1L add the following:
- 800mL distilled H2O
- 8g NaCl
- 0.2g KCl
- 1.44g Na2HPO4
- 0.24g KH2PO4
- 2ml Tween-20
- Adjust to pH 7.4 with HCl
- Add distilled H2O until final volume of 1L is reached

Blocking Solution

- 2g BSA (2%)
- 5mL Donkey serum (5%)
- 200uL Triton-X
- 100mgs of sodium azide (0.01%)
- Fill to 100mL with PBS-T



Additional figure: Representative image of muscle fibre borders stained with WGA and type II fibres stained with MHCII in green. Satellite cells (coloured arrows) stained with Pax7 in red and nuclei stained with Hoechst in blue. 20X magnification. Scale bar 50µm.



Additional figure: Representative image of muscle fibre borders stained with WGA and type II fibres stained with MHCII in green. Satellite cells (green arrows) stained with Pax7 in red. Muscle capillaries stained with CD31 in purple and nuclei stained with Hoechst in blue. 20X magnification. Scale bar 50µm.

Appendix E: Western blot

Protocol for Western blotting

1. Make up resolving gel
2. Make up proteins to 20 ul
    a. Add 5ul of Lamelin with 5% b-mecaptoethanol
3. Place in heating tray
4. Make up stacking gel
    a. Add it to the top of the resolving gel
    b. Insert comb
    c. Wait to set
5. Set up gels & tank
    a. Remove Vaseline from cassette
    b. Remove coms
    c. Place in tank with 1X running buffer
6. Load gels
    a. Add 30 ul sample per well and 5 ul ladder
7. Run gels for 2 hours at 75 volts
8. Transfer
    a. Cut a sheet of nitrocellulose and place it on top of a transfer sponge
    b. Place the gel on top of the transfer paper and another sponge
    c. Rinse in transfer buffer
    d. Run in biorad transfer for 20mins AMPS under 2A
9. Remove from transfer cassette and add panceau
10. Image Panceau and wash off with TBST 3 x 5 mins
11. Block with 5%milk in TBST for 90mins
12. Add primary antibody in 1:1000 5% milk in TBST at 4 degrees Celsius over night
13. Wash off with TBST 5 x 5mins
14. Add secondary antibody 1:10 000 in 5% milk in TBST
15. Wash off with TBST 5 x 5mins
16. Image using the BioRad

Recipes for solutions

Running buffer 10X

- 30.3g Tris (0.25M)
- 144g Glycine (1.92M)
- 10g SDS (10%)
- Make up to 1L with dH_2)

Transfer buffer 10X

- 30g Tris (250mM)
- 150g Glycine (1.92M)
- Make up to 1L with dH_2O
- For 1X transfer buffer: dilute 100ml 10X transfer buffer: 200mL Methanol: 700mL dH_2O

TBST 10X

- 60g Tris (500mM)
- 87g NaCl (1.5M)
- 10ml Tween (1%)
- pH to 8.3
- make up to 1L with dH_2)

Gel Constituents

Stain free resolving gel 12%

5.7mL	dH_2O
4.5mL	40% Acrylamide
3.75mL	1.5M Tris-HCL pH 8.8
150uL	10% w/v SDS
150uL	10% w/v APS
10uL	TEMED

Stacking gel 4%

4.84mL dH_2O

1mL	40% Acrylamide
2mL	0.5M Tris-HCL pH 6.8
80uL	10% w/v SDS
80uL	10% w/v APS
8uL	TEMED

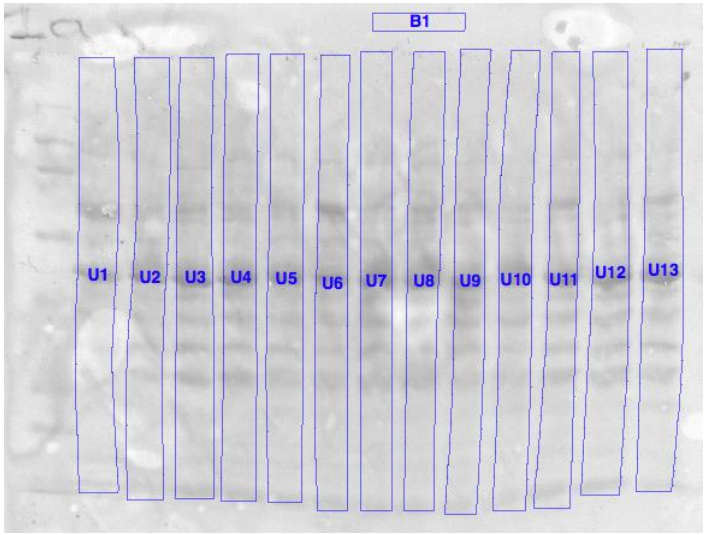
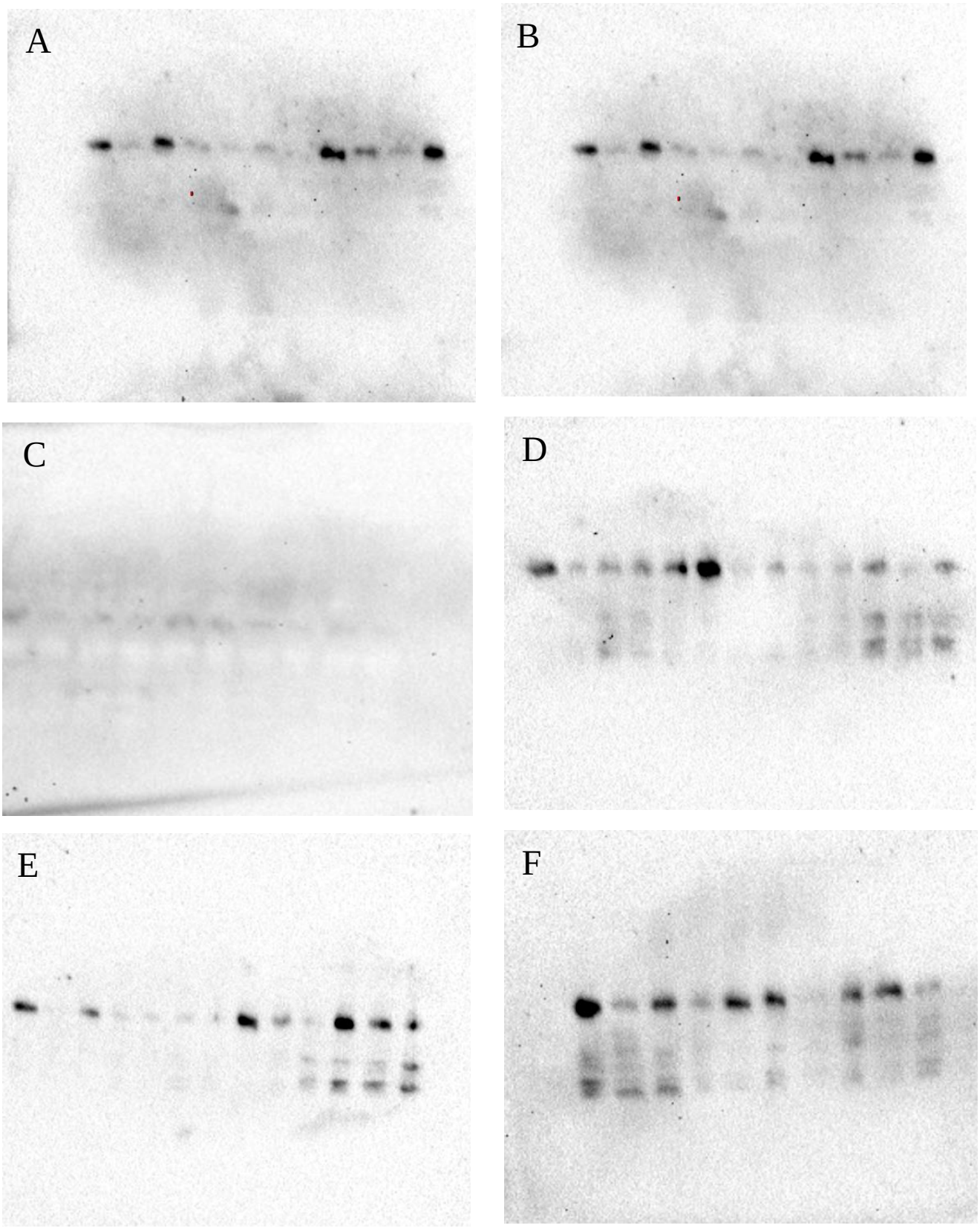


Image of the total protein content on the gel with Panceua staining



Unanalysed western blot images for gels (A-C) of myoD and (D-F) myogenin

Appendix F: Additional results

Table illustrating the muscle fibre morphological characteristics in response to 4 weeks of HIIT

		UPHILL		DOWNHILL	
	Fibre Type	**Baseline**	**4 Weeks**	**Baseline**	**4 Weeks**
CSA (μm^2)	I	$^{\#}$5510 ± 2080	$^{\$\#}$6221 ± 1188	$^{\#}$6543 ± 2111	$^{\$*\#}$8418 ± 1818
	II	$^{\#}$7804 ± 2030	$^{\$\#}$8238 ± 1958	$^{\#}$8342 ± 2629	$^{\$*\#}$11103 ± 2495
MN/F	I	$^{\#}$2.56 ± 0.6	$^{\#}$2.57 ± 0.4	$^{\#}$2.8 ± 0.1	3.1 ± 0.5
	II	$^{\#}$2.90 ± 0.6	$^{\#}$3.05 ± 0.4	$^{\#}$3.3 ± 0.2	3.3 ± 0.4
MND	I	$^{\#}$2228 ± 674	2517 ± 667	$^{\#}$2272 ± 327	$^{\#}$2713 ± 105
	II	$^{\#}$2807 ± 697	2710 ± 582	$^{\#}$2508 ± 1207	$^{\#}$3216 ± 215

Data expressed as Mean ± SD
* Significant effect of training $p < 0.05$
# Significant difference between fibre type within particular group and particular time point $p < 0.05$
$ Significant difference between groups for the particular fibre type at the particular time point $p < 0.05$

Table illustrating the satellite cell response to 4 weeks of HIIT

		UPHILL			DOWNHILL		
	Fibre Type	**Baseline**	**Acute**	**4 Weeks**	**Baseline**	**Acute**	**4 Weeks**
SC Cells per fibre	I & II	0.10 ± 0.02	0.10 ± 0.01	$^{\$}$0.10 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	$^{\$*}$0.29 ± 0.02
	I	0.12 ± 0.03	0.11 ± 0.02	0.11 ± 0.1	0.09 ± 0.01	0.10 ± 0.03	$^{\#}$0.16 ± 0.79
	II	0.12 ± 0.03	0.11 ± 0.03	$^{\$}$0.10 ± 0.91	0.11 ± 0.03	0.09 ± 0.01	$^{\#\$*}$0.33 ± 2.19

Data expressed as Mean ± SD
* Significant effect of training $p < 0.05$
# Significant difference in fibre type $p < 0.05$
$ Significant difference between groups $p < 0.05$

Table illustrating the muscle capillary response to 4 weeks HIIT

	UPHILL			DOWNHILL		
	Baseline	**Acute**	**4 Weeks**	**Baseline**	**Acute**	**4 Weeks**
C:F	1.7 ± 0.2	1.7 ± 0.2	$^{*\$}$2.6 ± 0.2	1.6 ± 0.3	1.7 ± 0.1	$^{\$}$1.7 ± 0.2
Capillary Density (x mm^{-2})	249 ± 39	243 ± 57	$^{*\$}$304 ± 42	217 ± 12	228 ± 7	$^{\$}$194 ± 44

Data expressed as Mean ± SD
* Significant effect of training $p < 0.05$
# Significant difference in fibre type $p < 0.05$
$ Significant difference between groups $p < 0.05$