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The Effect of Critical Limb Ischaemia on Adult Human Skeletal Muscle

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DECLARATION CONCERNING THESIS PRESENTED FOR THE DEGREE OF DOCTOR OF MEDICINE

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solemnly and sincerely declare, in relation to the thesis entitled:

The Effect of Critical Limb Ischaemia on Adult Human Skeletal Muscle

(a) That work was done by me personally;

(b) The material has not previously been accepted in whole, or in part, for any other degree or diploma.

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Abstract

Potential therapeutic avenues may emerge from understanding morphological adaptations of adult human skeletal muscle that result from critical issue ischaemia (CLI). There is little understanding of the role and limitations of satellite cells in repairing and regenerating ischaemic tissue. This study aims to show the pathognomonic changes that take place as adaptations to chronic ischaemia.

Patients (n=10) undergoing lower limb amputations for critical limb ischaemia were recruited to the study and gastrocnemius muscle biopsies were compared to those from control patients (n=10) undergoing long saphenous vein harvesting for coronary artery bypass grafting. Transmission Electron microscopy, histology, immunohistochemistry and Western blotting were used to assess the myogenic response to ischaemia. Any significant change in tissue morphology, morphometry and satellite cell number or activity was of interest.

There was significantly greater deposition of fibrofatty tissue, collagen and a loss of polygonal structure in CLI samples. Myonuclear number per fibre was not significantly different, neither was the occurrence of centrally occurring nuclei. All fibre types demonstrated significant atrophy except IIc. IIc hybrid fibres were more abundant in CLI samples ($p=0.0147$). Type I fibres displayed a proportionate rise by 2.4 fold ($p=0.0288$). Type IIx fibres were most susceptible to ischaemia with x6 fold reduction in number ($p=0.0039$) and greatest reduction in CSA ($p=0.0029$). Type II fibres showed greater fibre-size diversity.

The endothelial marker, CD31 and the haematopoietic stem cell marker, CD34 were more abundant in CLI ($p<0.0001$). There was over expression of the satellite cell marker pax7 ($p<0.0001$) and quiescent satellite cell numbers. MyoD, a marker of activated satellite cells is significantly reduced in ischaemia ($p<0.0001$).

These findings confirm active repair and regeneration in CLI tissue, but the indigenous response of muscle is inadequate for proper healing. These processes are disordered, with limited maturation of myogenesis.

Keywords: Ischaemia, critical limb ischaemia, satellite cells, myofibres, haematopoietic stem cells, myogenic precursor cells, plasticity, myogenesis

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LIST OF ABBREVIATIONS

ABC	Avidin-biotin-complex
ABPI	Ankle to brachial pressure index
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
Ang 1	Angiopoietin 1
Ang 2	Angiopoietin 2
ATP	Adenosine triphosphate
CABG	Coronary Artery Bypass Grafting
CDK	Cyclin Dependent Kinase
CDKI	Cyclin Dependent Kinase Inhibitors
CLI	Critical limb ischaemia
CVA	Cerebrovascular Accident
DAB	3, 3’-diaminobenzidine tetrahydrochloride
DAPI	4', 6-Diamidino-2-phenylindole
DPX	Distyrene, plasticiser, and xylene
eNOS	Endothelial nitric oxide synthase
EC	Endothelial cells
ECM	Extra-cellular matrix
EDTA	Ethylenediaminetetraacetic acid
Epo	Erythropoietin
EpoR	Erythropoietin receptor
FGF	Fibroblast growth factor
FIH	Factor inhibiting HIF
FITC	Fluorescein isothiocyanate
G-CSF	Granulocyte colony stimulating factor
H&E	Haematoxylin and Eosin
HGF	Hepatocyte growth factor
HIF	Hypoxia inducible factor
HMEC	Human microvascular endothelial cells
HREs	Hypoxia response elements
HRP	Horse-radish peroxidase

HSCs	Haematopoietic Stem Cells
iNOS	Inducible nitric oxide synthase
IGF – 1	Insulin growth factor – 1
IMG	Intussusceptive microvascular growth
JAK–2	Janus kinase–2
MAPK	Mitogen–activated protein kinase
MCAD	Medium-Chain Acyl-CoA Dehydrogenase
MEF2C	Myocyte enhancer binding factor 2C
METH	Metallospondin
M:F ratio	Microvessel to muscle fibre ratio
MHC	Myosin Heavy Chain
MMPs	Matrix metalloproteinases
MRFs	Muscle Regulatory Factors
MVD	Microvessel density
MyoD	Myogenic Differentiation Factor
NCAM	Neural Cell Adhesion Molecule
NGS	Normal Goat Serum
NHS	National Health Service
NO	Nitric oxide
OD	Optical density
PBS	Phosphate buffered saline
PECAM–1	Platelet endothelial cell adhesion molecule
PDGF	Platelet derived growth factor
PlGF	Placental growth factor
PMSF	Phenylmethylsulfonylfluoride
PTA	Percutaneous transluminal balloon angioplasty
PAOD	Peripheral Arterial Occlusive Disease
PVD	Peripheral vascular disease
rb-1	Retinoblastoma protein
RDU	Relative density unit
RGS	Regulators of G–protein signalling
ROS	Reactive oxygen species
SCs	Satellite Cells
SD	Standard deviation

SDF	Stromal derived factor
SMCs	Smooth muscle cells
TASC	Transatlantic Inter-Society Consensus
TEM	Transmission electron microscopy
TNF	Tumour necrosis factor
TRIS	Tris (hydroxymethyl) aminomethane
uPA	Urokinase plasminogen activator
VEGF	Vascular endothelial growth factor
VEGFR1	Vascular endothelial growth factor receptor 1
VEGFR2	Vascular endothelial growth factor receptor 2

Chapter 1 Introduction

1.1 Critical Limb Ischaemia

1.1.1 Chronic Limb Ischaemia: Natural History, Symptoms and Signs

Intermittent claudication, rest pain and critical limb ischaemia are due to the same process of atherosclerotic disease but of varying degrees, disrupting blood flow to the end organ which is the motor unit. These clinical manifestations collectively represent the spectrum known as peripheral arterial disease. Symptomology worsens proportionately with decreasing Ankle to Brachial Pressure Index (ABPI). Most patients with chronic limb ischaemia first manifest with pain in the calf when walking, particularly uphill, and this intermittent claudication of gastrocnemius occurs due to the large requirements of the muscle for oxygen and nutrient outstripping those supplied by atherosclerotic vessels. Relief from calf pain is typically achieved after a few minutes rest. Intermittent claudication may also affect the large and energy hungry gluteus and thigh muscles when limited by poor blood flow.

Critical Limb Ischaemia causes rest pain which occurs in the toes and foot when the leg is elevated. Sufferers often sleep in chairs or hang the affected limb out of bed as gravity assists the impaired flow of arterial blood. Invariably the disease progresses from rest pain to ulceration and or gangrene within weeks. Ulceration or gangrene may sometimes be the first signs of chronic limb ischaemia as patients may have poor exercise tolerance or be bed bound. Rest pain, ulceration and/ or gangrene is termed as critical limb ischaemia (ABPI <0.4 or <50mmHg) whereby the limb is threatened. (1-3)

1.1.2 Epidemiology of Critical Limb Ischaemia

Critical Limb Ischaemia (CLI) is defined as the presence of rest pain, ulceration and/ or gangrene in a limb of a duration of over 2 weeks and it affects between 500-1000 patients per million annually. Around 30% require a major amputation within the first year after diagnosis and a further 10% will lose their limb over the next two years. The presence of CLI is a marker of high mortality and morbidity as it infers the presence of cardiac or neurovascular disease. Patients have a poor life expectancy, with a 25% mortality rate within a year of diagnosis or over 50% over 5 years. Patients with CLI are six times more likely to die from a cardiac event within ten years compared with non ischaemic patients (4;5;5-8).

Prevalence of arterial disease increases with age and the incidence of peripheral arterial disease in men <50 years old is 1.5%. In 55-74 year old males this rises sharply to 5% and in men aged 75 and over, more than 20% are affected. In younger age groups, there is a 2:1 male to female ratio but this almost equals out in older age groups. Critical limb ischaemia represents 1-3% of the whole spectrum of peripheral arterial occlusive disease.(4;4)

Approximately 30% of patients with symptomatic disease will die within 5 years and about 78% die within 15 years compared to 22% in asymptomatic patients (5;6). The severity of the disease is such that it and its risk factors must be treated aggressively as early as possible. Without intervention and revascularisation, most CLI patients would lose their affected limb within 6 months. Treatment with major surgery carries a 5-6% 30 day mortality rate (7). 5.1 major amputations per 100 000 population are performed a year (8).

1.1.3 The Economic Impact of Critical limb Ischaemia on the NHS

CLI is a significant drain on the National Health Service, to the tune of £300m per year. For those patients suffering from peripheral arterial disease who are at grave risk of losing limbs, lifestyle and independence, there are limited options available. Therapeutic angiogenesis is as yet unproven, but in principle the option of manipulating the processes of angiogenesis and myogenesis via injections, or direct administration of stem cells would be far preferable to invasive management (9). Operative interventions including amputations carry their own significant risks, complications and long term sequelae.

The cost of maintaining care for those patients with chronic ulceration is impressive. In the UK, chronic wounds represent a significant burden to patients and the NHS and their impact on quality of life is well documented (10). Around 200,000 patients in the UK have a chronic wound of which a significant proportion are arterial in nature. The total cost of maintaining these wounds is estimated at around 2.3bn–3.1bn per year (11). It is presumed that there are around 64,000 individuals with active foot ulceration at any one time and 2,600 amputations are carried out annually as a direct result (12).

The total cost of a major amputation far outstrips that of PTA or reconstructive surgery. This is due in part to the lengthy inpatient stay, the cost of physiotherapy, adaption of or re-housing, drug costs, occupational therapy support and equipment and out of hours care (13). The total cost of managing patients with an amputation is in the order of £200 million/ year excluding social care (14;14;15). The average medical cost of an amputee in their first year is in the order of £3000-12000. This compares to mean angioplasty costs approximately £6600 and open arterial bypass around £6800 (16;17).

1.1.4 Risk factors for Critical Limb Ischaemia

The risk factors for chronic limb ischaemia are the same as for any atherosclerotic disease. Management of atherosclerosis assesses these risk factors and aims to reduce their systemic effect through behavioural or pharmacological interventions. The management of established or symptomatic peripheral atherosclerotic disease is discussed later.

1.1.4.1 Smoking

'Smoking is the greatest single cause of preventable illness and premature death in the UK.' 500, 000 deaths of adults aged 35 and over are attributable to smoking in England and Wales (18;19).

Around 26% of the British population aged 16 and over are smokers. This equates to around 11 million with an equal number of ex-smokers. 25% of British men smoke compared with 23% of women and they also tend to smoke more heavily. The highest prevalence is seen in the 20-34 year old age bracket (Information Centre, Independent NHS Special Health Authority, Smoking, Drinking and Drug Use among Young People in England in 2006). Recent estimates place the real cost of smoking to the NHS at around £1.5 billion annually.

Smokers have a 1.7 - 5.6 fold increase in the development of atherosclerosis and according to the American Heart Association, Heart Disease and Stroke Statistics of 2007, the relative risk of developing symptomatic peripheral arterial disease is up to tenfold. The Framingham Study clearly underlines the importance of smoking cessation.(1;2;2)

1.1.4.2 Diabetes

Diabetes mellitus has a similar impact on the chance of developing atherosclerosis, the risk increasing by 3.5 fold in men with glycosuria. In older women with glycosuria, the protection thought to be conferred by oestrogens is eradicated and their risk rises by 8.6 times, to the same level as that of older men. The AHA Atherosclerotic Vascular Disease Conference 2004 quoted "diabetes adversely modifies the clinical course of PAD [Peripheral Arterial Disease] and is the most common cause of amputation in the United States, accounting for 45% to 70% of all non traumatic amputations." (6;6;7;20)

1.1.4.3 Abnormal Lipid Profile

The relative risk in causing peripheral arterial disease or claudication, carried by each 10mg/dL rise in total cholesterol over normal levels is about 1.1 times. Elevated levels of triglycerides and Low Density Lipoproteins (LDLs) with reduced levels of High Density Lipoproteins (HDLs) are seen in many patients with atherosclerosis and this dyslipidaemia contributes further to the development of atheroma and thus PAD (21;22)

1.1.4.4 Hypertension

Hypertension only increases the risk of PAD by 10%. Treating and decreasing hypertension rates in a 9 year trial held in the United States lead to lower rates of bypass and amputation. The Framingham Study data encourages the requirement of patients to control hypertension (1). Up to 5% of hypertensive patients have PAD at presentation and it has been

recommended that the optimal treatment targets a blood pressure of 140/85mmHg or 130/80mmHg if there is concomitant renal disease or diabetes exists (2;23;24).

1.1.5 The Clinical Diagnosis of Critical Limb Ischaemia

CLI is a disease characterised by finding the following; poor pulses, ulceration, necrosis, alopecia of the limb, muscle atrophy, skin changes, delayed capillary refill and dependant hyperaemia (positive Buergers test); all of which are indicative of poor tissue perfusion. To quantify this objectively, establishing the ankle brachial pressure index (ABPI) is crucial. An abnormal ABPI is indicative of PAD and a value of 0.90 is 90% sensitive and 95% specific (3). Low ankle pressures of <50-70mmHg and toe pressures of <30-50mmHg tend to result in ischaemic ulcers. Transcutaneous oxygen tension can often be used to quantify CLI, with a $TcPO_2$ of <30-50mmHg being indicative (25)

Imaging techniques to confirm the diagnosis and identify areas of stenosis or occlusion include Duplex ultrasound, CT Angiography, MR Angiography and Percutaneous Angiography.

1.1.6 The Management of Critical Limb Ischaemia

The management of critical limb ischaemia is limited and culminates in three endpoints. Treatment can be symptomatic and essentially palliative, restoration of in line flow to the lower limb can be attempted, or perigenicular amputation could be offered. Therapeutic angiogenesis has not yet been established as a viable treatment option.

1.1.6.1 Lifestyle Changes, Medical Management and Palliation

Simple treatment of atherosclerosis requires eradication of risk factors. Lifestyle changes are necessary; regular exercise, cessation of tobacco smoking and an improved diet are all proven to be of benefit. Smoking cessation reduces not only the risk of progression of PAD to CLI but also the likelihood of myocardial infarction (1). Numerous trials have shown statins similarly are of great benefit in reducing cardiac events and coronary artery related deaths by between 24-34% (22).

The management of peripheral arterial disease requires the use of an antiplatelet (13). Aspirin inhibits cyclo-oxygenase and therefore thromboxane A manufacture, as well as prostaglandin I_2 formation, by its irreversible acetylating actions on COX-1 and –2 (26). Thromboxane A receptors, located throughout the vascular endothelia and present on the surface membrane of platelets, are responsible for causing their aggregation. Clopidogrel inhibits platelet adenosine diphosphate receptors. The CAPRIE (Clopidogrel versus Aspirin for the Prevention of Recurrent Ischaemic Events) Trial comparing the two drugs demonstrated an even greater benefit for peripheral arteriopaths compared to cardiac patients taking clopidogrel in that the drug reduced their number of cardiovascular events by 7.3% (22;27). Ulcer management

without further treatment to blood flow can be ongoing in the community, with opiate pain control where required in those patients unsuitable for in line reconstruction.

1.1.6.2 Therapeutic angiogenesis

The study of angiogenic inhibition has been the target of cancer research for some time, as cancers require a blood supply to grow. The body of research that has resulted from this has also lead to a better understanding of pro-angiogenic factors which has resulted in elucidating which of those agents which may be administered to improve peripheral circulation. Still in its infancy, evidence for therapeutic angiogenesis is encouraging but far from conclusive.

This strategy may be beneficial for those patients who cannot undergo major surgery or even PTA to avoid limb loss (28). The form of administration or vector that these agents require is one of the greatest rate limiting steps in the development of therapeutic angiogenesis. Attempts have been made by directly introducing recombinant proteins into the bloodstream; or even via gene transfer. These methods allow a systemic response and are easy to administer (29) but do have implications for co-existing disease, i.e. cancer; large intramuscular bolus injections into affected muscle are currently being investigated and these have the additional advantage of requiring less recombinant protein and remaining in the local environment for sufficient time periods (30). Viral delivery, while potentially accurate, can cause harm in the form of inflammation and secondary necrosis (31). DNA-plasmids used in animal models have been relatively successful suffer with low tissue permeability due to poor cellular penetration (32;33).

Hepatocyte growth factor (HGF), Vascular Endothelial Growth Factor (VEGF) (34)and Fibroblast Growth Factor (FGF) have all been tested as pro-angiogenic agents in CLI patients (35;36). Mostly only small trials have been conducted and therefore no conclusive outcomes have been reached. Studies have not been standardised in terms of administration protocols, nor measurable outcomes and taken with the often low numbers examined in these studies, results have rarely been impressive enough to warrant further investigation (35;36).

The Therapeutic Angiogenesis with FGF-2 for Intermittent Claudication (Traffic) Trial 2002 was a blinded randomised, placebo-controlled Phase II trial which recruited 190 patients with refractory angina or intermittent claudication. The FGF arm was given intra-arterial infusions of 30mcg/kg of recombinant basic FGF on day 1 or day 1 and day 30. There was found to be a small but significant improvement in ABPI at day 90 following a single dose administration of FGF-2 (37). The third phase of the TAMARIS has recently shown that in 525 patients given intramuscular injections of FGF, there was no perceivable improvement in mortality or major morbidity rates (38).

It is likely that a combination of pro-angiogenic agents or administering them in conjunction with stem cells will yield better results than a single agent alone. A great deal of work regarding their mode of delivery remains. The correct dosages need to be established and the optimal duration of treatment must be established. Reducing the side effects associated with pro-angiogenic agents, more efficient gene transfer with greater local expression of agents and less systemic effects is required.

1.1.6.3 Interventional Techniques to Improve Inline Arterial Flow

1.1.6.3.1 Percutaneous Angioplasty

Open surgery and percutaneous angioplasty are currently the options available to the surgeon to combat CLI. Percutaneous methods of treating PAD take the form of balloon angioplasty, the placement of stents within the arterial lumen, or subintimal canalisation. Aortoiliac stenoses can be treated effectively with PTA and stenting; 5 year patency rates are in the order of 60-80%, however reconstructive surgery does offer a better outcome. It has been recommended that longer stenoses and occlusions are treated with open surgery and endovascular treatment of dense calcifications carries a high risk of rupture or distal embolisation. In treating short stenoses of the superficial femoral or popliteal arteries, angioplasty has a 5 year patency rate of 70% but much lower rates in longer stenoses or occlusions >4 cms (39-42).

1.1.6.3.2 Bypass Surgery

Depending on the position of the stenosis or occlusion in the patient's vasculature, co-morbidities, life expectancy and the risks vs the benefits of the procedure itself must all be taken into account when devising an appropriate surgical intervention. Aorto-iliac disease is best treated with an aorto-bifemoral bypass graft although other options such as ilio-femoral, femoro-femoral or even axillo-(bi) femoral bypasses maybe considered. These offer the benefits of lesser trauma to the patient but have lower patency rates due to the length of the grafts involved. In more distal disease, where there is adequate run off, femoral-popliteal or

femoral-distal bypass is required and the greatest patency results are gained through the use of vein grafts rather than PTFE or other synthetic grafts (41-43)

Those patients undergoing infrainguinal bypass have a poor post operative outcome, with 5 year primary patency of grafts particularly grim if the primary presenting complaint was gangrene (33%) compared to 52% for ulceration or rest pain. The 5 year cumulative limb salvage rates were in the order of 59% for gangrene, 87% for ulceration and 83% for rest pain (44).

1.1.6.4 Perigenicular Amputations

There is a further subset of patients in whom reconstructive surgery should not be considered and for whom amputation is the most suitable procedure. These include those with poor run-off vessels, fixed flexion deformities, advanced diabetic neuropathy, extensive necrosis and/ or infection of the foot. Common sense dictates that bed bound patients or those nearing the end of life due to advanced biological (rather than chronological) age, sufferers of dementia who are unable to make an informed decision regarding an amputation are all groups who would do poorly after reconstructive surgery and who may benefit from a primary amputation in the presence of CLI (16).

Around 20% of patients having had a below knee amputation and 50% of patients after an above knee amputation do not return to functional mobility. Almost a third of these patients require a major amputation of the other limb within 5 years and the mortality rate for these patients in the same time frame is around 50% (16). Although the decision to perform a major

amputation is never taken lightly, sometimes an early decision regarding level of amputation is beneficial, especially considering that a failed infrainguinal bypass may result in an above knee amputation when a pre-bypass below knee amputation may have been adequate (45).

1.1.7 The Pathogenesis of Atherosclerosis

Endothelial dysfunction is present in those patients without obvious atherosclerosis but with risk factors for the disease (46;47) and is necessary for atherosclerosis to develop (22;48-51). Platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), transforming growth factor (TGF)–β, thrombin, and angiotensin II are potent mitogens produced by the activated platelets, macrophages and dysfunctional endothelial cells that characterise early atherosclerosis and result in local inflammation and thrombosis at sites of endothelial disruption due to platelet activation and adherence (46;49;52). Reduced NO, activation of platelets and increased vasoconstrictors create an environment more likely to lead to atherosclerosis and thrombosis (48). Atherosclerosis is a multifactorial disease involving the processes of inflammation, lipid, endothelial and platelet cell dysfunction, altered clotting and thrombus formation, extracellular matrix remodelling, oxidative stress, vascular smooth cell activation and genetic factors (53). Low Density Lipids, LDLs, penetrate the endothelium and are oxidised by macrophages and smooth muscle cells whilst growth factors and cytokines recruit further cells such as monocytes, foam cells and more smooth muscle cells. The formation of a plaque is initiated and this disrupts endothelial cell function. Lipids are a major component in the laminar formation of atherosclerotic plaques and form one of the initiating layers accumulating on damaged endothelium (48;54). Platelets become pro-thrombotic and adhere to the exposed subendothelium after the rupture of a plaque and the release of lipids, collagen, von Willebrand and tissue factor into the circulation (22;48-51).

1.2 The Structure of Normal Adult Human Skeletal Muscle

1.2.1 The Structure of Normal Adult Human Skeletal Muscle and the Classification of Myofibres

Adult human skeletal muscle is composed of differing fibre types each surrounded by the basal lamina and located within a fascicle bordered by the scaffolding of a collagenous connective tissue or endomysium, (see **Figure 1**). The differences in fibre type lie within the contractile abilities and fatigue resistance of the myosin heavy chains of the myofilaments which make up each myofibril and in turn each myofibre, (see Table 1). As each particular muscle varies considerably in its function, its composition of fibre type is thus different due to the variation of Myosin Heavy Chains (MHCs) expressed (55).

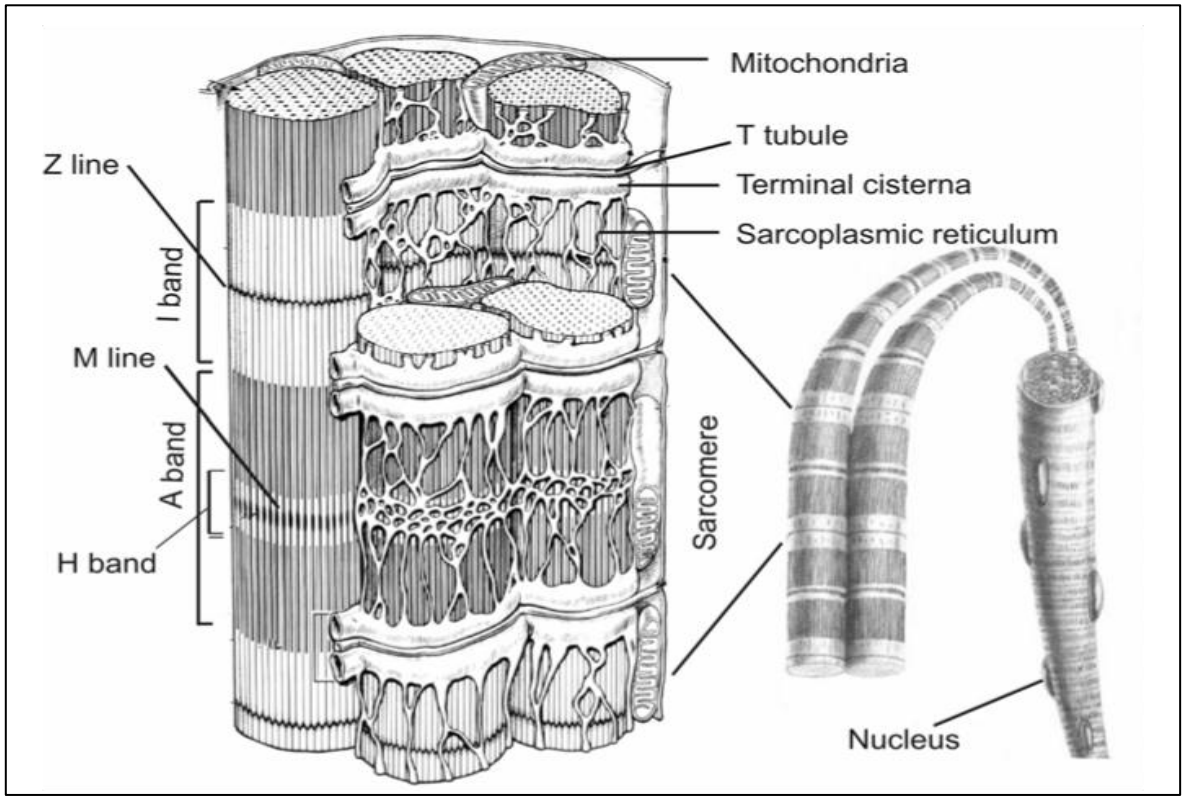


Figure1: Adapted from Rogers 1983 (56) Schematic Diagram Showing Adult Human Skeletal Muscle Architecture, A myofibre on the right of the diagram is composed of a

syncytial arrangement of myofibrils within a fascicle, surrounded by an endomysium layer which is an invaginated extension of the endomysium which covers the whole muscle. A myofibre is composed of repeating units called sarcomeres which extend from Z line to Z line (also called a Z disc) to which actin and titin (or connectin) molecules are bound.

Muscle fibre type can be classified according to the expression of the Myosin Heavy Chain (MHC) which in turn governs the properties of the fibre, as shown in **Table 1**. A single myosin molecule comprises of heavy and light chains of variable number. The head region of myosin contains the ability to cross react with actin, hydrolyse ATP and thereby generate the contractile function of skeletal muscle by causing a sliding action along actin filaments. In mammalian tissue, myosin heavy chains exist in at least eight different myosin heavy chain isoforms (57) and are detailed in **Table 2**. This expression is under the control of muscle innervation and altering the nerve supply of a motor unit will change its MHC isoforms (58;59).

Table 1: Classification of Myofibres By Myosin Heavy Chain Isoform and Their Relative Characteristics:			
	Slow or Type I (Soleus)	**Fast or Type IIa (Gastrocnemius)**	**Fast or Type IIx**
Colour	Red	Red	White
Maximum Shortening Velocity	Slow	Fast	Very Fast
Motor neurone	Small	Large	Very Large
Resistance to Fatigue	High (Slow Fatigue Resistant)	Intermediate (Fast Fatigue Resistant)	Low (Fast Fatiguable)
Activity	Aerobic	Anaerobic	Anaerobic
Force production	Low	High	Very High
Mitochondrial density	High	High	Low
Capillary density	High	Intermediate	Low
Metabolism	Oxidative	Oxidative Glycolytic	Glycolytic
Major storage fuel	Triglycerides	Creatine Phosphate, Glycogen	Creatine Phosphate, Glycogen
Calcium Uptake In SR	Slow	Fast	Fast
Myofibrillar ATPase Activity	Low	High	Very High

1.2.2 Myosin Heavy Chain Expression in Human Gastrocnemius

In human adult lower limb skeletal muscle, three different muscle fibres have been identified according to their myosin heavy chains. These were originally identified and classified according to their histochemical and oxidative capabilities and therefore their reaction to myofibrillar adenosine triphosphatase (mATPase) and since have been confirmed by electrophoresis.

Table 2: Comparative Presence of Myosin Heavy Chain Isoforms in Rat and Human Species.

Myosin Heavy Chain Isoform (MHC)	Isoform Present(+) or Absent (-)			
	Rat	Human	Adult Human	Adult Human Skeletal
α cardiac (Chr. 14)	+	+ v	+	-
β cardiac/ I (Chr. 14)	+	+	+	+
MHC IIa (Chr. 17)	+	+	+	+
MHC IIx (Chr. 17)	+	+	+	+
MHC IIb (Chr. 17)	+	-	-	-
MHC-IIm (Chr. 17)	+	-	-	-
Embryonic (Chr. 17)	+	+	-	-
Neonatal (Chr. 17)	+	+	-	-
Extraocular (Chr. 17)	+	+	+	-
Only three isoforms are found in the skeletal muscles of the lower limb of humans; Types I, IIa and IIx (57)				

Gastrocnemius in particular lends itself to study due to its accessibility for biopsy methods (open or percutaneous) and displays the greatest morphological change due to the fact that this is the commonest site of intermittent claudication symptomology (60). Most studies have demonstrated that 50-60% of gastrocnemius fibres are type I and 40-50% are type II although, as in many other muscles, the level of cross section through a muscle dictates the composition (55) and indeed gastrocnemius has two heads of insertion and these have a slightly different function (61). Depth of sample from the surface of organ is also important.

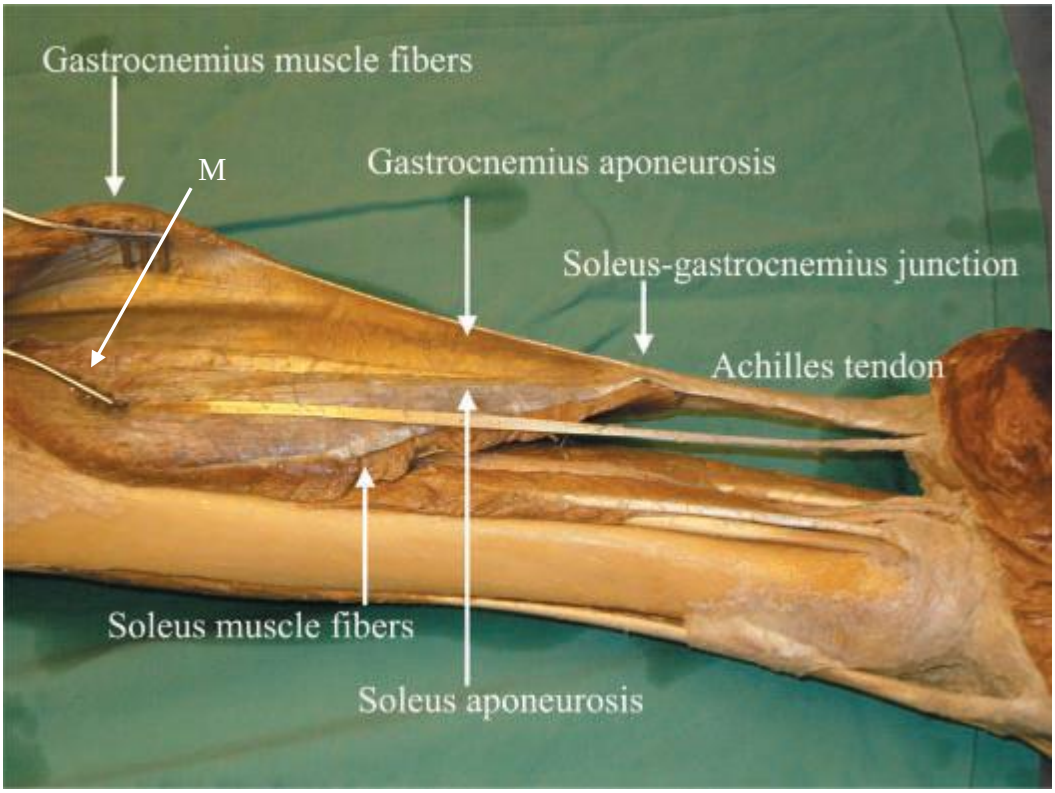


Figure 2: Cadaveric Dissection of the Posterior Aspect of the Lower Leg Showing the Position of the Medial Head of Gastrocnemius [M]. *Adapted from Bojsen-Moller et al 2004* (61)

Smerdu et al. (62) looked at gastrocnemius samples in a small cohort of young, healthy and sedentary individuals (not immobile) and showed the relative proportions of the different MHC phenotypes present within the muscle and these are indicated below in Table 3:

Table 3:Relative MHC Isoform Proportions in the Gastrocnemius Muscles of Sedentary Men

MHC Isoform	Proportion (%)
Type I	69.7% ± 2.0
Type IIa	24. 1% ± 1.7
Type IIx	4.3% ± 0.7
Hybrid Fibres (Type IIc)	1.8% ± 0.6

Smerdu demonstrated a change in fibre composition in that disuse of muscle allows for a prevalence of the type I fibre type in gastrocnemius. The medial head of gastrocnemius has an equal proportion of type I to type II fibres. Variables such as age, exercise tolerance and disuse affect the proportionate mixture of fibre types through plasticity or fibre remodelling, discussed later.

Those muscles that cross a single joint contain a relatively high proportion of slow myosin or function as anti-gravity muscles are likely to be most affected by immobilisation. In fact, a primate study has shown that muscles were more susceptible to the effects of immobility in a well described order: soleus; plantaris, vastus intermedius, vastus lateralis, gastrocnemius, tibialis anterior, rectus femoris (63).

Table 4: Characterisation of Rat Vs Human Tissue: Common Antibodies Used for phenotyping and the Corresponding MHCs they stain		
	Species and Fixation	
	Rats	Humans
	Frozen	Paraffin
Antibody	**Fibre Types Stained**	
sc75	All but I	-
BAD5	I	-
M8421	I and I/IIa	I and I/IIa
BFF3	IIb	?
BF-35	All but IIx	?
RTD9	IIx and IIb	?
sc71	IIa	?IIa and ?IIx
A4.74	IIa	IIa and IIx

Table 4: Myosin Heavy Chain Antibodies and Their Use in Tissue Samples; Rat frozen tissue is the most frequently characterised whereas human paraffin embedded tissue has the least range of commercially available antibodies for MHC analysis. (-) denotes a negative stain and (?) denotes no reported use in paraffin embedded tissue.

Reliable immunohistochemistry in paraffin embedded sections is currently limited to delineating fibres into fast and slow, or type I and type II, whereas frozen tissue can be more easily typed and all fibre types can be identified. The advantage of using paraffin embedded tissue is however that its architecture is better preserved and therefore it lends itself to better morphological analysis.

The different types of ATPase histochemistry require separate sequential slides are prepared, treated individually and analysed in a sequential way to identify fibre type. Behan et al (2002) demonstrated that simple differentiation of type I and type II subgroups was possible using alkaline phosphatase (ALP) conjugated antibodies and removed the need for several

sequential slides but was further limited by the availability of commercially available ALP conjugated antibodies. Like ATPase histochemistry however, it was also unable to reliably demonstrate hybrid fibres (64) unless sequential slides were utilised and therefore has limited value alone.

1.2.3 The Blood Supply in Normal Gastrocnemius:

Anatomical studies show a difference in the blood supply of gastrocnemius and soleus; the former having capillaries running parallel to the muscle in a straight fashion, whereas the richer capillary supply of soleus tends to be more tortuous and have many more branches. This differing capillary structure was first commented on in humans by Sjostrom (65) and more recently in rats by Erzen (66) via stereology. Capillary density and topography adapted to the needs of the aerobic and predominantly oxidative fibres of soleus (67). Andersen showed that age alone does not significantly affect the number of capillaries present in muscle (68).

1.2.4 The Role of Satellite Cells in the Repair and Regeneration of Normal Skeletal Muscle

The existence of satellite cells in skeletal muscle, first identified and named by Alexander Mauro in 1961, was initially discovered in the sartorius muscle of the frog (69). They were described under electron microscopy, as a distinct population of cells, lying above the muscle fibre sarcolemma but beneath the fibre basal lamina, and accounting for up to between 3-6% of visible myonuclei seen (70). Their structure clearly intimated that they were completely different to muscle cells, being mononucleate, with large nuclei and a relatively small amount of independent cytoplasm. Mauro also pointed out that under light microscopy, these would therefore appear merely as "a peripheral muscle nucleus proper" (69) although Watkins and Cullen claimed even under light microscopy that satellite cells are distinguishable from myonuclei due to their smaller size (71). It is reported that a quiescent satellite cell in mammalian skeletal muscle is around 25 μms in length, 4 μms in height and 5 μms in width (72).

Satellite cells are the myogenic precursor cells of postnatal muscle responsible for the repair and regeneration of muscle fibres in adult tissue, either by fusing together and forming new fibres or incorporating themselves into damaged muscle cells. They are modulated both by inflammatory cells and locally damaged tissue and the release of such mediators as nitric acid (NO) or IGF-1(73).

The basal lamina also appears to play its part and in animal models where muscle has been crushed (74), frozen (75) or injected with toxins (76), the satellite cell response appears to follow two paths, apparently dependant on whether the basal lamina is intact. If this is the

case, activated satellite cells contained within this layer migrate to the site of injury in the same muscle fibre via chemotaxic stimuli (77;78). Activation of cells has been reported at around 6 hours post injury and within 24 hours displays significant mitosis (78). The satellite cell then merges with the damaged myofibre and helps repair or regenerate either myofibre material or contribute to the myonuclei (79;80). In injuries where there is disruption of the basal lamina, satellite cells are able to migrate from adjacent myofibres by projecting across tissue bridges initiated from an out pouching process of the satellite cell itself (77;81). Little is known of how the cellular machinery alters and morphology of the satellite cell changes as the cells perform this active process (82).

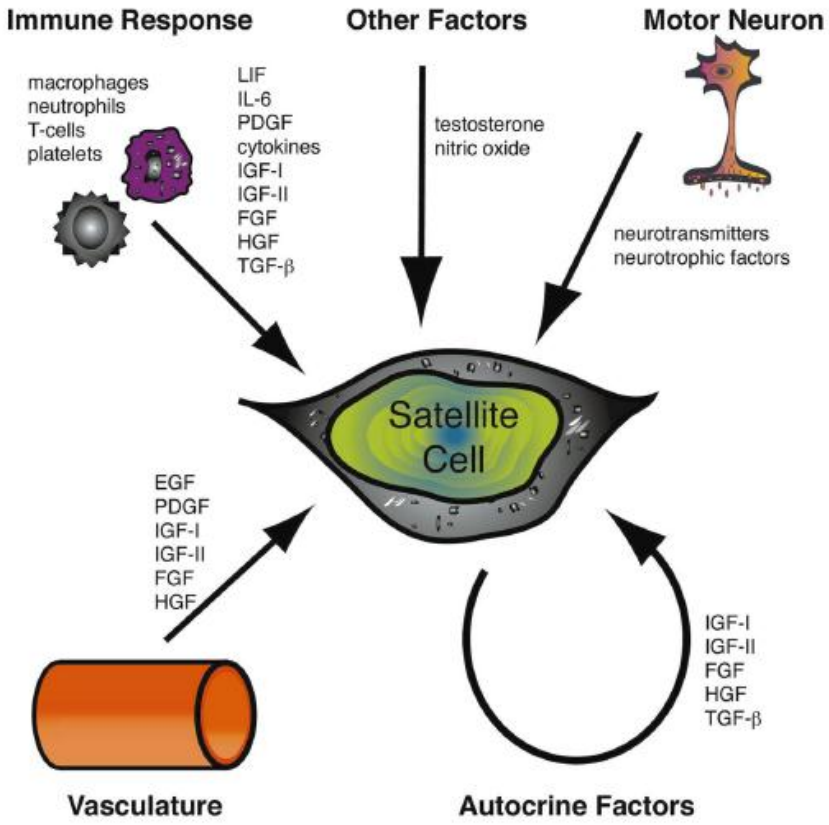


Figure 3: Factors responsible for modulating the activity of satellite cells (83). *Adapted from Hawke and Garry 2001*

Destruction of satellite cells in adult small mammals via gamma irradiation leads to retarded muscle growth (77;84;85). In animal experiments, it has been shown that endurance exercise, or exercise induced ischaemia, increases the proportion of satellite cells and other myonuclei (86). The activation of satellite cells in response to such training ensures that the proportionate number of myonuclei/ fibre remains constant allowing continuing control despite the increase in fibre size and number. This is reflected in the increasing number of myonuclei seen in exercise induced cellular hypertrophy, while conversely being lost during atrophy (87).

1.2.5: Satellite Cell Repair and Regeneration:

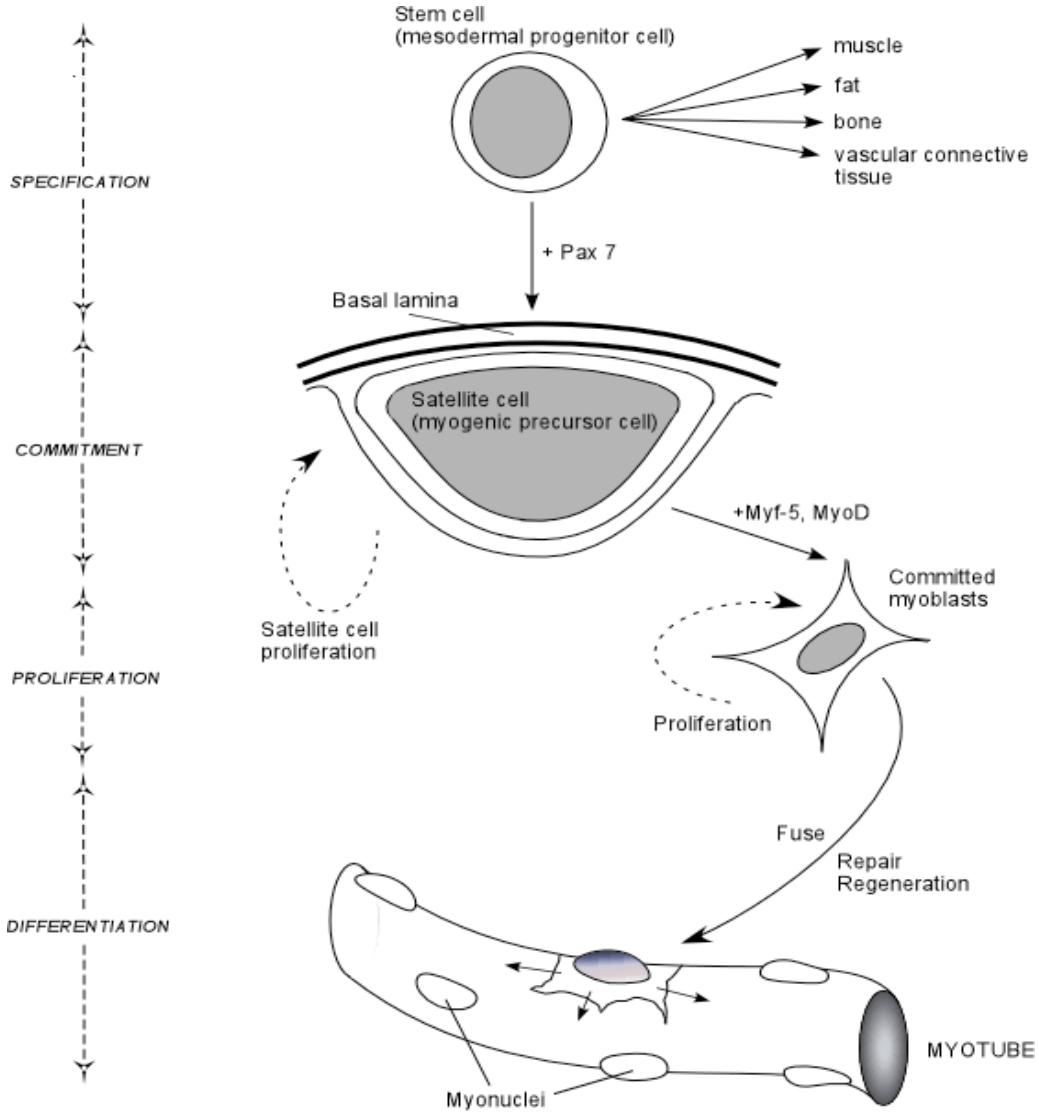


Figure 4: Schematic diagram demonstrating the role of the satellite cell, as a bipotent stem cell, contributing to hypertrophied myofibres or self renewing the satellite cell population.

1.2.6 Satellite cell numbers and regenerative potential:

In normal muscle, the number of satellite cells is stable in relation to the area of the muscle under its sphere of influence and accordingly larger myofibres have more satellite cells associated with them (88). Cornelison (1997) et al. found no obvious difference in satellite cell numbers around any particular fibre type (89). *In vivo* studies demonstrate a decline in cell population numbers with age (90-92). It has been reported previously that satellite cell proliferation is increased in the following: denervation, endurance exercise, low-frequency electrical stimulation, stretch, testosterone and immobilization and decreased with age. There are few reports of their behaviour in chronic limb ischaemia (83;93-97).

It is clear that nerve degeneration and the reduction in sarcoplasm to nucleus ratio leads to volume loss (98;99) but despite this, the number of myonuclei per fibre remains constant. In the soleus muscle of rats that Hikida et al (1998) found no statistical evidence to support the reduction of myonuclei number/ fibre following atrophy caused by 10 days of space travel on the shuttle Endeavour (100), indicating that the satellite cells tightly controlling the myofibre domains and replacing nuclei material had been unaffected by altitude or weightlessness.

It is likely that satellite cell numbers vary in any given muscle and in any given subject. Sajko suggests inconsistencies in the literature are due to irregular distribution and low numbers of identifiable satellite cells in normal tissue (101). It has been demonstrated that with advancing age the domains governed by the myonuclei enlarge, satellite cell numbers decrease and the tight control seen in the repair and regeneration of more youthful tissue is lost (88). Numbers of satellite cells are generally thought to decline throughout the aging process, although why this should be remains unclear. Electron microscopy has been used to

show that there is no significant difference between the number of satellite cells in young compared to older sedentary human subjects (102). Other studies using different techniques numbers disagree, with some reporting a decrease in satellite cell number in aged tissue (103;104). It has been shown that while the numbers of cells present is important, their regenerative potential that may be affected, for instance becoming reduced in old age (101;105). The number of times a satellite cell *in vivo* can undergo cell repair and or regeneration using daughter progeny is a contentious issue. Some groups report great success at proliferating human skeletal muscle-derived stem cells *in vitro* and at achieving multiple daughter progeny of multiple mesodermal cell types, up to 18 times from a mean donor age of 63 years in one study (106). Although the number of divisions that a satellite cell may undergo is finite, some models have suggested that age alone does not limit the regenerative potential of satellite cells (107;108). Lipofuscin accumulation or limited mitogenesis eventually causes delayed or reduced satellite cell function.

1.2.7 Myogenesis and the Muscle Regulatory Factors

The complex interaction of the Cyclins, (i.e. Cyclin D3), CDKs (Cyclin Dependant Kinases) and the CDKIs (Cyclin Dependant Kinase Inhibitors) controls the myoblast cell cycle (109). These CDKIs (Cyclin dependant Kinase Inhibitors) especially p21, greatly expressed in G_1, establish the post-mitotic state during myogenesis (after withdrawal from cell cycle and before differentiation), and essentially halt the cell cycle by blocking the action of the Cyclins and CDKs. p21 stops the phosphorylation of pRb by inhibiting CDK action, allowing accumulation of its active form, thereby continuing the permanent arrest of the cell cycle (109-112). MyoD greatly influences the cell cycle through enhancing the actions of p21, pRb and Cyclin D3 (110-112).

Proliferating myoblasts also require myf5 for differentiation (110;111;113) and then later MyoD. Expression of myf5 occurs in G0 and MyoD in the G1 stage of the cell cycle (110;113). MyoD and myf5 ensure permanent withdrawal from the cell cycle and induce specific muscle gene expression. These MRFs confer upon myoblasts their myogenic fate. Skeletal muscle differentiation is characterised by terminal withdrawal from the cell cycle, muscle specific gene expression and the formation of myotubes. This occurs at the expense of further cell proliferation (114). Lassar and Valdez showed that after myf5 and MyoD expression, myogenin is next required for cell differentiation (115). The MRFs also initiate the fusion of myotubes later in the process of myogenesis. Kitzmann et al. demonstrated on mouse cell lines in vitro that these events must occur in sequence for normal myogenesis.

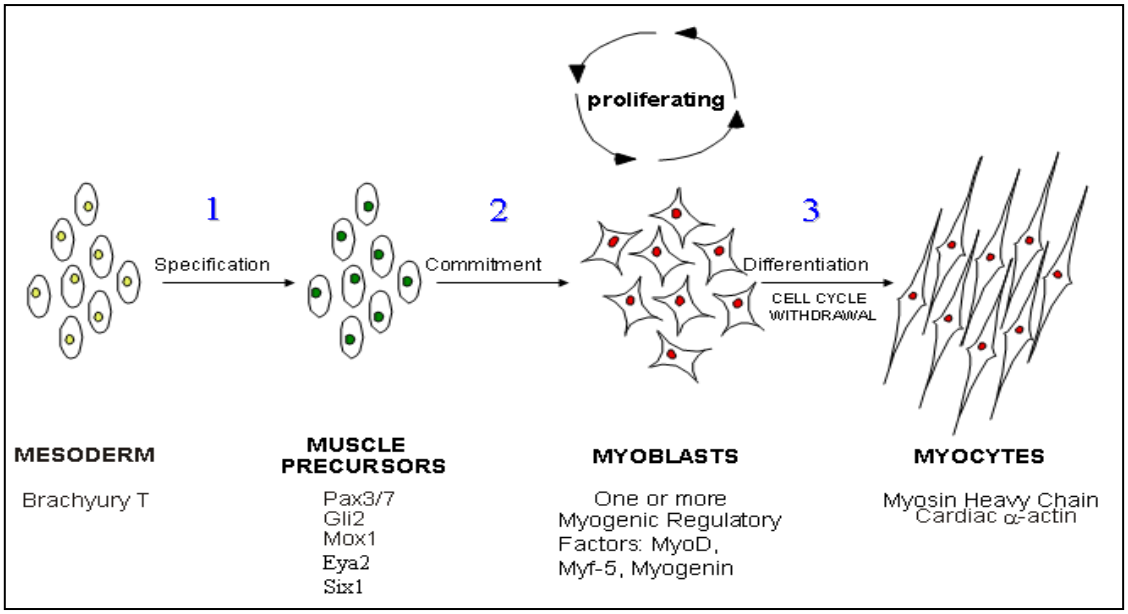


Figure 5: Stages of Normal Myogenesis in Human Skeletal Muscle

After muscle gene expression is up-regulated within the myoblast, fusion occurs into multinucleated primary myotubules. These cluster together under a basal lamina, whilst the recruitment of further myoblasts will lead to the development of secondary tubules as well as satellite cells. This is stimulated by nitric oxide. The processes of proliferation and differentiation cannot take place concurrently and therefore once myoblast proliferation has taken place differentiation may resume only once proliferation signalling pathways are switched off.

The muscle regulatory transcription factors (MRFs), MyoD, myogenin, MRF4 and Myf5 are able to dictate a skeletal muscle phenotype to non-specific progenitor cells. Myostatin acts through pax7 to switch on satellite cell driven regeneration and switch off satellite cell renewal (116). Proliferating myoblasts express myoD and myf5 before they enter

differentiation as do satellite cells (SCs) undergoing differentiation into myofibres or satellite cells. (111;113;113)

As long as the basal lamina remains intact in the injury, satellite cells fuse with each other to form myotubes, which then mature into a new myofibril or fuse with an existing one (78). The regeneration process is identical irrespective of the aetiology of damage (117).

1.3 Remodelling of Skeletal Muscle in Response to Ischaemia

The term “plasticity” has been in use since the late 1950s to describe the adaptive change in human skeletal muscle fibre type, remodelled to meet physiological or pathological demand. Plasticity therefore is a normal characteristic of adult skeletal muscle. Physiological demands (endurance training, high altitude and advancing age) and pathological conditions such as ischaemia directly affect skeletal muscle architecture and MHC composition, altering postural control, strength and function, myogenesis and local nerve and blood supply. Muscle can be pre-conditioned with an adaption of fibres, extracellular matrix and angiogenic response to a given stimulus and due to the presence of myogenic precursor cells, can also be repaired and regenerated to specific requirements. Studying muscle morphology allows the plastic response to be quantified.

The pathognomonic changes due to nerve damage are the most striking in ischaemic conditions. The presence of (commonly type II) angular fibres, indicates denervation and in some studies was seen in 100% of PAD samples. Fibre specific clumping, indicative of reinnervation, has been demonstrated in around 50% of PAD biopsies (65;68;118-120). Hedberg demonstrated that most reinnervated bundles appear to be type I (120).

The effects of denervation not only result in angular fibres but also cause atrophy, affecting both fast and slow muscle fibres. There is an apparent reduction in fibre cross sectional area as well as muscle force. Plasticity of fibres similar to that seen in immobile or sedentary patients occurs with an overall shift from fast to slow MHC. This direction of plasticity is also seen secondary to critical ischaemia which preserves type I fibres and type II fibres preferentially atrophy or necrose (44;62;102).

Plasticity is influenced locally by satellite cells (SCs) under the control of the Muscle Regulatory Factors (MRFs) (60;121;122). These stem cells are probably the only significant source of new myoblasts in the adult tissue, but the contribution of bone-derived haematopoietic stem cells (HSCs), vascular progenitor cells, or interstitial cells has not been fully evaluated (96). In younger adults, this activity is well regulated and controlled, but this tight co-ordination (123) breaks down with age (90;124) and it has been proposed that individual satellite cells behave independently or do not respond to normal cues and may contribute to heterogeneous MHC phenotypes, perhaps accounting for much of the conflicting data available. Braun et al. demonstrated an increase in satellite cell derived myoblasts following freeze injury and cardiotoxin induced skeletal muscle damage in mice as well as an upregulation of the pax7 gene expression in these myoblasts (125). It is thought that satellite cells are also modulated by the fibrocyte/ adipocyte progenitor cells that are seen in greater numbers in ischaemic muscle. These bipotent FAP cells have been shown to reduce haematopoietic stem cell differentiation and have an inhibitory effect on myogenesis (126;127). It has been postulated that these cells have not only a regulatory effect but may be interchangeable with adipose progenitors, potentially able to form multi-mesenchymal line, including haematopoietic stem cells, myogenic precursors, fibrocytes and adipocytes (126;127).

The co-ordinated action of SCs is required for the process of plasticity and is also responsible for fibre shift in tissue. Quiescent satellite cells are activated by the presence of the damaged basal laminar structure and specific extra cellular signals such as Wnt, IL-6 and the IGFs, with inhibition of terminal differentiation by the Notch system; leading to myoblastic proliferation but differentiation is inhibited.

It has been determined that an IGF-1 splice variant known as Mechano Growth Factor (MGF) is first expressed by damaged myoblasts and it is this that first activates satellite cells (128). The active satellite cells or myogenic precursors will finally contribute to mature post-mitotic myofibres and may be stimulated by the release of IGF-1ea another splice variant of IGF1 (128-130). The cascade that fulminates in the activation of satellite cells is as (128) follows: inflammatory reaction, followed by oedema, degeneration and necrosis with ensuing phagocytosis of such material and finally the regeneration of muscle fibres (93).

These processes were formalised into the following pathway (131;132)

- Hypoxia
- Release of chemo attractive factors.
- Vasodilatation
- Leucocyte adhesion
- Neutrophil and macrophage migration and induced necrosis
- Activation of satellite cells

Myoblasts closely interact with and are under the control of local inflammatory factors and stromal cells. Inflammatory monocytes may be recruited to aid myogenesis (129;133). Chronic dysfunction from fatty degeneration due to white adipose cell and fibrocyte accumulation, reduces the ability of the muscle to self-renew. These cells derive from myogenic precursor cells that fail to undergo normal myogenic differentiation due to disease processes or age related limited mitogenesis or lipofuscin accumulation (129).

1.4 Myogenesis In Ischaemia

In CLI there is a shift from oxidative phosphorylation to glycolysis and the response to diminished reserves of ATP (adenosine triphosphate) is either the arrest of the cell growth or programmed cell death, apoptosis.

There appears to be a threshold whereby mild hypoxia (6% O_2) actually increases cell turnover and survival, as Di Carlo et al. showed this is similar to O_2 tension within exercising muscle (134). Their work demonstrated a response to greater hypoxia (~1% 0_2) was found in C2C12 mouse myoblast cells in vitro, which block Myf-5. MyoD expression was reduced due to the increased rate of degradation by the ubiquitin-protease pathway. Thus the recruitment of myogenic cells was reduced; the cell cycle arrested in the G_1 phase and terminal differentiation inhibited (134).

Hypoxia causes the inhibition of myogenin, p21 and active hypophosphorylated pRb. The induction of pRb by MyoD is a key event (proven both in vivo and vitro) in both arresting the cell cycle and causing differentiation. Thus blocking MyoD leads to the inability to withdraw from the cell cycle and terminal differentiation (56;164;165;170;173). Genetic and biochemical evidence indicates that MyoD and Myf5 establish the myogenic lineage whereas myogenin promotes terminal differentiation (111;134;135).

These responses to hypoxia are mediated by the "master mediator" HIF-1, a transcription factor itself induced and stabilised by hypoxia (110-112;136). HIF-1, among other roles, acts to promote the actions of p21 and p27 (CDKIs discussed earlier) to arrest the cell growth, as well as up regulating expression of tumour suppressor factor p53. The action of CDKI p27

and myostatin cause an increase in the active hypophosphorylated pRb, again causing the inability to withdraw and leading to arrest of differentiation (110-112;136).

The reduction in the expression of the MRFs caused by hypoxia is reversible, with levels of MyoD, myf5 and myogenin restored once oxygen tension is restored. Functional pRb is required for this arrest, not p21. Hypoxia only temporarily arrests the cell cycle, with normal myogenesis recurring after restoration of normoxia (110-112;136).

1.5 Fibre Type Shift and Atrophy in Chronic Limb Ischaemia

There is a complex interplay between the susceptibility of differing fibre types to ischaemic conditions and the active repair and regeneration of these fibres performed by satellite cells. Fibres display plasticity by switching phenotypes, expressing two or more different MHCs along the course of their length (spatial transition). These 'hybrid or jump fibres' (137) identifiable through immunohistochemical techniques are much more prevalent than previously thought (68;119;138-140).

Even in the normal aging process, there is a related fibre type change resulting in a gradual temporal and spatial transition from fast to slow MHC isoforms; IIx → IIx/a → IIa →IIa/I →I (141), with concurrent preferential loss of susceptible Type II fibres and the marked atrophy of all those that remain. The opposite shift is seen in bed rest and disuse (140) and the fact that age and disuse are both common concomitants with ischaemia clearly confounds morphometric analysis to such an extent that there is no consensus on the direction of fibre type shift in chronic ischaemia.

In conditions such as critical limb ischaemia it is likely that the more susceptible Type II fibres either undergo apoptosis, atrophy or display hybrid features as they change into type I fibres (119;138) but this has not been fully elucidated. The phenotype changes that occur in the ischaemic patient are similar to those seen in age related change but to a much greater degree (118).

Calf muscle mass is reduced in the elderly and in PAD (60;139;142). It has been recognised for some time that muscle fibres atrophy and via the activation of proteolytic enzymes such as the calcium-activated neutral protease family of calpains can be triggered to auto-digest by high levels of intracellular calcium (143).

Connective tissue infiltration increases by 30-50% in ischaemic muscle. This is both fibrous tissue, presumed secondary to limb disuse, but also likely a result of the activation of inflammatory and cellular mediators (120;144). Studies by other groups (145) have suggested hypoxia causes an upregulation of the Transforming Growth Factor-β (TGF-β) family which is pro-fibrotic and increases deposition of collagen in other progressive muscular dystrophies and inflammatory myopathies It is likely that a similar process occurs in CLI (42;146). TGF-β1 tends to be observed near areas of myofibre damage and necrosis, high TGF-β2 mRNA and precursor protein levels have been observed in regenerating muscles after strain injury and particularly at the end of motor units as well as in fusing satellite cells and myotubes, although levels have been observed to decrease as the fibre matures (147).
TGF-β3 has also been observed in those damaged regions where macrophages were active in phagocytosing necrotic tissue. It appears that pax7 over expression (116;148); and a member of the TGF- β family known as Growth Differentiation Factor 8 (GDF8) or myostatin may

both act to inhibit the Muscle Regulatory Factors and therefore inhibit myogenesis and myogenic differentiation (149) although recent evidence suggests that while myostatin may inhibit myofibre differentiation and hypertrophy, it is unlikely to affect satellite cell function (150;151). In this way, the MRFs commit satellite cells to their myogenic fate, cause their proliferation but hinder normal differentiation leading to muscle weakness and fibrosis. TGF-β also recruits smooth muscle cells into the capillary walls and leads to the overgrowth of the extra cellular matrix. (152;153)

1.6 Angiogenic Remodelling in Skeletal Muscle in Chronic Ischaemia

All mammalian cells need oxygen and nutrients to survive and are never more than the diffuse limit of oxygen, or 200μms, away from a blood supply that can deliver such requirements. In order for tissue to grow, this blood supply must increase proportionately and it does this through vasculogenesis, the recruitment of vascular endothelial precursor cells in the embryo, or angiogenesis, the formation of new blood vessels. The process of new blood vessel formation is in constant flux under the influence of pro and anti angiogenic factors but the normal balance between the two groups of molecules leads to no net gain of vessel formation (154-157).

Circulating haematopoietic stem cells derived originally from endothelia or bone marrow respond to chemotaxic signalling from molecules such as platelet derived growth factor and can be directed to contribute to angiogenesis (158). With the correct stimulation, these cells are thought to be capable of repairing other mesodermal tissues including skeletal or cardiac muscle or even nerve cells (106;157;159;160).

Nitric oxide synthesases, VEGF and its receptors, angiopoietins through the Tie system, fibroblast growth factors, matrix metalloproteinases and collagen are all products of independent hypoxia induced gene action (136;161;162). VEGF and basic fibroblast growth factor (bFGF) are both mitogenic for capillary endothelial cells, with the former also increasing endothelial permeability and possibly stem cell recruitment and survival (163;164). Both factors are essential for angiogenesis and are seen in elevated levels in calf and thigh muscles of CLI patients (164-167).

Atherosclerotic induced ischaemia leads to an increased vasculogenic and angiogenic response, but arteriogenesis is impaired as is further maturation of new vessels (162). One of the key mediators of this hypoxia induced activity is HIF-1α, a DNA binding factor (136;157;162;168). As HIF-1α is upregulated, it is amplified by other growth factors, TGF-β, VEGF, angiopoietin, CXCR4, VCAM1 and Endothelin-1 stimulate angiogenesis through the sprouting, bridging or intussuception of mother vessels (136;157;162;168). HIF-1α has been co localised with CD31, a marker of endothelial cells (169). Interestingly, VEGF and VEGFR-2 are seen in greater concentrations with increasing degrees of ischaemia or more atrophic myofibrils (166;170).

Capillarisation of muscle tissue in ischaemia has been well reported (94). The vast majority of papers have looked at the capillary number per fibre (C: F) as well as the number of capillaries around a muscle fibre (CAF). Using both methods exclude bias due to any atrophy of fibres due to ischaemia. Most morphometric studies show significantly more capillaries present in ischaemic tissue (171;172), some authors have even suggested a preponderance of capillaries around type I fibres (65;173).

Specific endothelial marker studies have revealed frequent and frustrated attempts at capillary maturation in ischaemic tissue. An increase is seen in all immature endothelial cell subtypes tested for: CD34- immature haemopoietic stem/ satellite progenitor cells and vascular endothelial cells; CD31- all continuous vascular endothelium, including arteries, arterioles, venules, veins, non sinusoidal capillaries and lymphatic vessels; PAL-E - capillaries, venules and small and medium sized veins. Expression of α-SMA, a marker of pericytes seen in mature capillaries, is not increased. This indicates a positive primary angiogenic response in hypoxia, however, it is clear that maturation of these novel vessels is lacking (171;174).

Chapter 2 Hypothesis

Critical limb ischaemia causes altered muscle morphology clinically manifesting as pain, tissue loss, decreased exercise tolerance, poor balance, impaired proprioception, muscle atrophy and weakness. Therapeutic interventions commonly fail and advanced cases eventually necessitate amputation for symptom control. Stimulating the angiogenic pathway in CLI has been shown to be ineffectual, reducing the energy requirements of the end organ or increasing the effectiveness of satellite cells may provide an effective strategy in treating CLI. Little is known about the behaviour of satellite cells in the critically ischaemic leg. Greater understanding of myofibre behaviour in CLI and manipulation of multipotent stem cells resident in skeletal muscle may provide clues for a therapeutic strategy.

The hypothesis of this work holds that there is an adaptive response shown by skeletal muscle to chronic ischaemia, resulting in marked morphometric changes and tissue remodelling. Abnormalities are more pronounced in critical ischaemia, leading to greater dysfunction. The stem cell response to ischaemia is poorly coordinated and is insufficient to repair or reverse ischaemic damage resulting from CLI.

The aims of this study are as follows:

1) To describe the morphological changes exhibited by human skeletal muscle in response to critical limb ischaemia through the use of both light and transmission electron microscopy

2) To quantify those changes morphometrically.

3) To examine the adaptive mechanisms of skeletal muscle; in particular the response of resident stem cells to critical limb ischaemia.

Chapter 3 Materials and Methods

3.1 Patients

3.1.1 Ethical Approval and Consent

Ethical Approval was approved through the local ethical committee and allowed harvesting of human skeletal muscle from patients recruited through the Royal Free Hospital, Hampstead and the London Heart Hospital, Marylebone, (see Appendix A). All patients were provided with an information sheet explaining the muscle biopsy procedure and the purpose of the study (**Appendix A, Information Sheet**). Informed consent was obtained (**Appendix A, Consent form**). The Research reference number was 29-2000.

3.1.2 Patient Demographics

Ten patients undergoing perigenicular amputations for CLI were recruited as the study group and ten patients undergoing CABG with no evidence of PAD were recruited as the control group. Patients undergoing CABG who suffered with intermittent claudication, rest pain, ulcers, gangrene, oedema, peripheral vascular disease including varicose veins or lymphoedema, vasculitides or autoimmune disease, or myopathies were excluded. In those patients undergoing amputation for CLI, those patients with auto-immune disease or myopathies, venous disease or lymphoedema, acute ischaemia or advanced necrosis extending near to the medial belly of gastrocnemius were also excluded. Muscle biopsies

were taken from the medial head of gastrocnemius from both groups of patients via an open surgical approach described later (**Section 3.3**).

The patients recruited for this study were comparable for age and other characteristics, except for the presence of peripheral vascular history, namely lower limb arterial insufficiency.

Table 5: Patient Demographics and Co-morbidities

Ischaemic n= 10 vs Non-Ischaemic n= 10

	Ischaemic	Control	Test and Significance
Age	73.60 ± 2.405 N=10	73.20 ± 1.965 N=10	χ^2 ns
Sex	8M:2F	9M:1F	χ^2 ns
Dyscrasias	2	2	Fishers ns
Diabetes	6	5	χ^2 ns
Heart Disease	7	10	χ^2 ns
CVA/ TIA	2	1	Fishers ns
Cholesterol	8	10	χ^2 ns
Hypertension	8	9	χ^2 ns
Renal	2	1	Fishers ns
AAA	2	1	Fishers ns
Smoking history	9	8	χ^2 ns

Table 5: Summary of the patient groups and their co-morbidities: none of the control patients had symptoms of peripheral arterial insufficiency. Otherwise the cohorts were comparable for age, sex, blood dyscrasias (disorders including thrombocytopaenia), diabetes and heart disease including arrhythmias, angina or valve disease. Renal disease indicates the presence of chronic renal failure and renal artery disease, CVA/TIA also includes any intracranial bleed. All patients with hypercholesterolaemia were taking a statin. Any current or past history of smoking was counted as equally significant for the purpose of the study. The Fishers Exact Test was used to compare the two cohorts where $n<5$ and the χ^2 test where $n>5$.

3.1.3 Sample Collection and Preparation

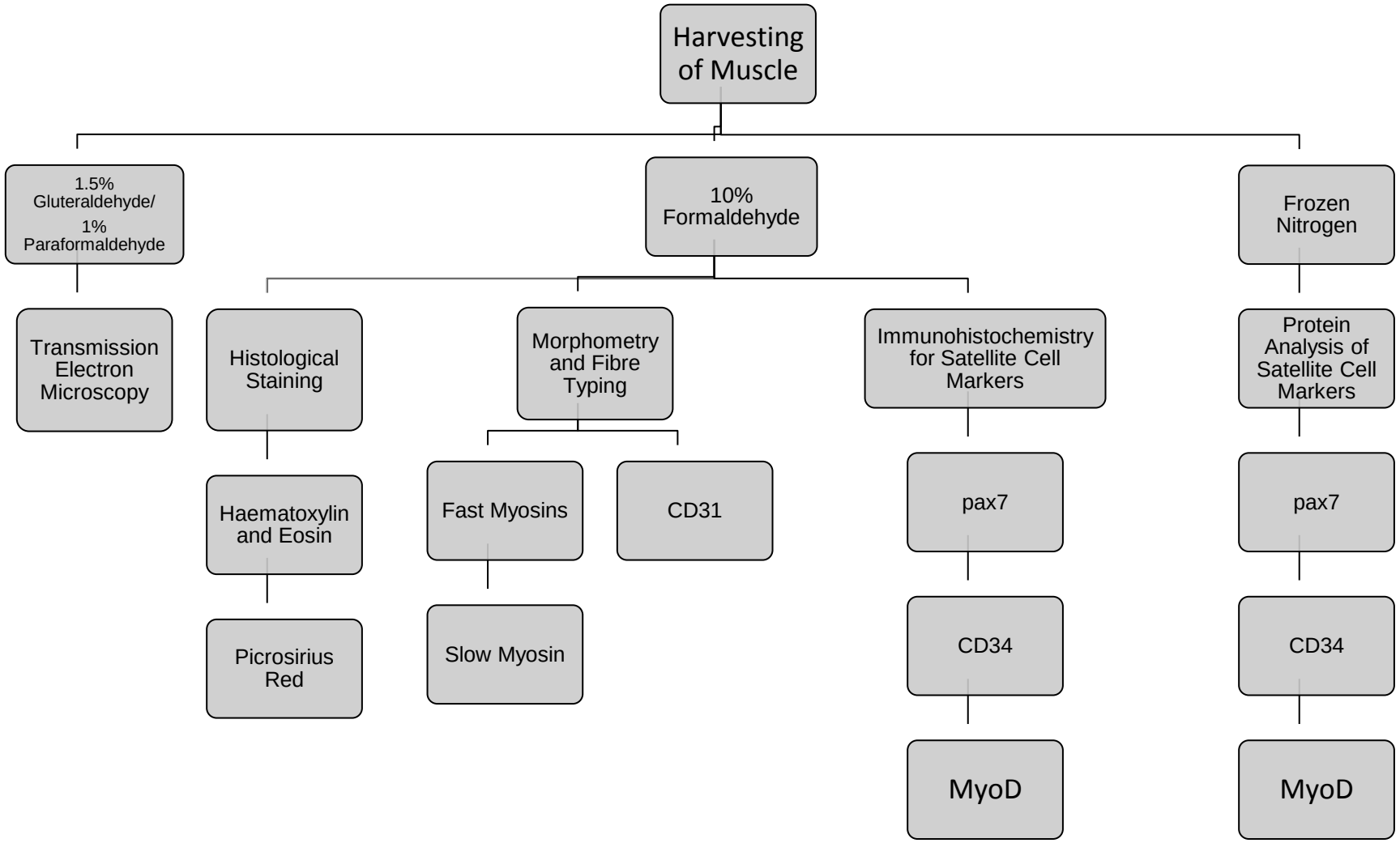
Patients undergoing perigenicular amputations for CLI or long saphenous vein harvesting for use in coronary artery bypass grafting were consented pre-operatively with attention paid to complications arising; such as bleeding, bruising or infection. This was in fact a minor procedure as the intended operation in both sets of patients was much more invasive and carried larger risks with greater tissue resection. No complications arose due to this study.

Tissue harvested was approximately $5mm^3$ in size and taken from the medial head of the gastrocnemius muscle. Samples were somewhat larger in those patients undergoing amputation due to the availability of whole muscle. The same area or level of the medial head of gastrocnemius was harvested as it is known that just as in other human skeletal muscles; the gastrocnemius is composed of different myosin heavy chains at different levels or depths through the muscle (55;98). To therefore avoid this variation confounding data collection, the samples were extracted from exactly halfway along the medial head of the gastrocnemius muscle for every patient.

In both sets of patients, these samples were taken well away from any areas of ulceration or oedema and these biopsies were divided into three sets of tissue. Muscle biopsies were processed as follows (91):

- immersed in formaldehyde to be paraffin embedded and used for histological, morphological and immunohistochemical analysis under light microscopy
- snap frozen in liquid nitrogen and stored at -80ºC for protein analysis
- 1% paraformaldehyde/ 1.5% glutaraldehyde in phosphate buffer for transmission electron microscopy (TEM) processing.

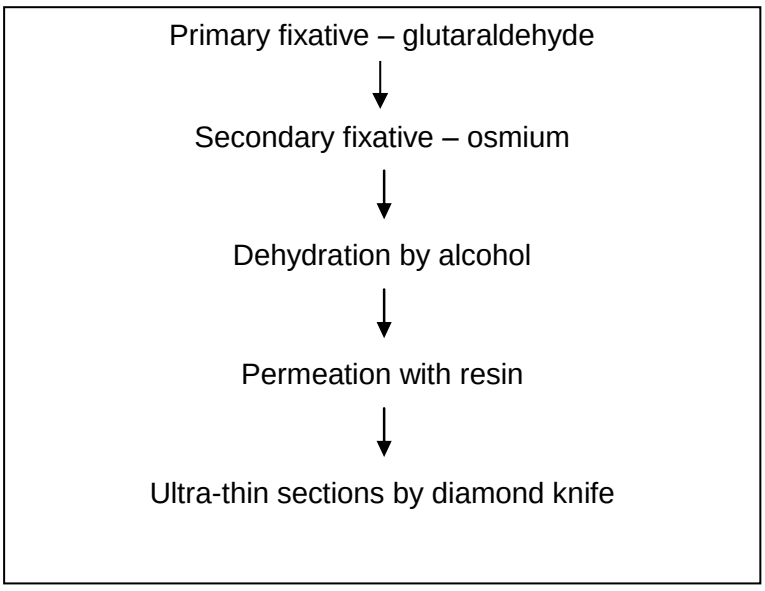
<u>Figure 6:</u> Schematic Diagram Demonstrating Use of Samples



3.2 Preparation methods of skeletal muscle for transmission electron microscopy (TEM)

The transmission electron microscope (TEM) uses electrons much as a light microscope uses light but whilst the latter is limited by the wavelength of light, the TEM using electrons is able to define objects at over x1000 greater resolution. The images viewed through the TEM are picked up via electromagnets and by adjusting these, the user can magnify images and pick up even greater detail. Limitations occur due to the electromagnets inability to focus the electron rays to a single focal point and increased refraction of light rays occurs, affecting the sharpness of an image, in much the same way that light behaves when passing through a lens. This is called spherical aberration. The electron beam must be passed through a vacuum so there is no deviation caused by the beam colliding with air. Similarly, the sample to be analysed must be dehydrated to avoid interference by the water particles contained within it and fixed in glutaraldehyde to avoid damage to the tissue. Preparation of tissue was performed to a standard protocol detailed below:

Figure 7: Method of Tissue Preparation for the TEM



3.2.1 Preparation of skeletal muscle tissue for TEM

Muscle biopsies were immediately placed in 1.5% glutaraldehyde/ 1% paraformaldehyde in PBS overnight and stored at -80ºC before further preparation as detailed below (91).

3.2.2 Fixation of Tissue for TEM

Although glutaraldehyde stabilises proteins within samples, and so preserves architecture, it does not fix lipids for which a secondary fixative must be utilised. Osmium tetroxide was used in this study to fix lipid membranes in the muscle samples which after dehydration with alcohol and infiltration with resin allow for the sample to be sliced with an ultra tome or diamond knife at a thickness of 60 nanometres. These ultra thin sections allow the passage of the electron beam and better resolution than the light microscope can obtain.

In this study, immediately after harvesting of tissue, small pieces of gastrocnemius were immersed in 1.5% paraformaldehyde/ 1.5% glutaraldehyde in 0.1M PBS pH 7.4 overnight. Post fixation was achieved with 1% osmium tetroxide and 1.5% potassium ferricyanide for 12 hours after thorough washing. The tissue was dehydrated through different concentrations of ethanol and then left in 50% epoxy monomeric resin 50% ethanol overnight before finally being completely preserved in 100% resin and placed in a 70^{o}C for 12 hours.

The final block, embedded in plastic was then stained with 1% toluidine blue and 0.2% pyronine in 1% boracic stain. A diatome was used to further slice 60 nm ultra thin sections of the prepared block and positioned on a copper mesh 3.05mm diameter x 0.7mls thick. A further stain of uranyl acetate in ethanol was applied for 5 minutes to the section followed by

lead acetate for 5 minutes. After a further wash with deionised water, the sections were placed within the Philips CM120 transmission electron microscope and image capture was performed at various magnifications, with note made of muscle architecture, satellite cell and mitochondrial number and characteristics.

3.3 Preparation of skeletal muscle for histological and immunohistochemical staining

Biopsies were placed immediately into Cellstor pots containing 10% neutral buffered Formalin (Cellpath UK Ltd) and stored at room temperature until further processing was carried out.

For the purposes of light microscopy, formalin fixed tissue was embedded in molten wax and applied to a cassette after orientating the tissue so that muscle fibres were perpendicular to the face of the block to be cut by microtome. The wax was cooled and the ensuing block was placed on a microtome positioned over a water bath. Sections were cut at a thickness of 3μms and great care was taken to apply these at the same orientation on polylysine coated slides (VWR International, UK). These were stored until use and dewaxing is described in the relevant sections (175-177).

Paraffin embedded tissue was used primarily due to the better preservation of tissue, especially due to morphometric analysis and it has been reported previously that cryoprocessing leads to greater fibre size discrepancy than is seen in paraffin embedding of the same tissue samples and is therefore less reliable (178).

Following an initial Haematoxylin and Eosin stain to determine orientation of tissue, Picrosirius Red and the following antibodies were used on sequential slides; Slow Myosin, Fast Myosin (A4.74, sc71 and BF-35), Embryonic Myosin, Neonatal Myosin, , CD31, CD34, pax7, myoD. The protocols used for each and the method of data collection and slide analysis are detailed below in Tables 8 and 9.

Negative controls for immunohistochemistry to show results were not due to non-specific staining, were performed without the primary antibody in each case, as detailed in previous studies (179). Except for this omission, the rest of the outlined protocols were followed as detailed below. These control samples were performed with runs of the final optimised positive samples to ensure no background staining ensued due to the application of the secondary antibody or chromagen as a result of the immunohistochemical process.

3.3.1 Histological staining of skeletal muscle

A simple haematoxylin and eosin stain was initially performed on each sample for the purposes of correctly orientating the skeletal muscle samples. Cross sections perpendicular to the muscle fibre were desirable for morphometric analysis. Those samples found to be at the incorrect orientation were realigned and cut again until correctly orientated (180;181)

3.3.1.1 Haematoxylin and Eosin Protocol

A simple Haematoxylin and Eosin (H&E) stain was used to determine correct tissue orientation, crucial for morphometric analysis of tissue. Sections were deparaffinised and rehydrated through xylene, ethanol and finally placed into deionised water, the whole process taking 10 minutes. Harris Haematoxylin (Surgipath Europe Ltd, Peterborough, UK) was poured through filter paper to remove any oxidised particles and sections were placed in a dry rack for immersion in the filtered solution for three minutes to stain nuclei Slides were rinsed for 5 minutes in tap water allowing development of the haematoxylin. Differentiation of sections was achieved with 1% acid ethanol for thirty seconds, before several immersions in blueing solution arrested differentiation to give a near colourless background. The slides were shaken dry and dipped into 1% aqueous eosin counter stain for 30 seconds before being dehydrated through ethanol and xylene for ten minutes.

Sections were mounted with distyrene (a polystyrene), a plasticiser (tricresyl phosphate) and xylene (DPX resin, BDH Gurr®). Slides were examined under a light microscope and pictures taken with Zeiss Axioskop 2 mot (Carl Zeiss, Gottingen, Germany).

3.3.1.2 Picrosirius Red Protocol

Picrosirius Red staining was used first to determine overall architectural differences in the muscle sections biopsied from the ischaemic and control muscle and to determine any difference in nuclei number and collagen deposition (182). Sections of both control and ischaemic muscle tissue were dewaxed and rehydrated through serial immersion in xylene and ethanol (100%). Staining with Celestine blue for 1 minute and haematoxylin for 5 minutes was performed to stain cell nuclei and the slides were next washed for 10 minutes in deionised water. Picrosirius Red (Direct Red 80, picric acid 1.3% in water, Sigma Aldrich) stain was applied to the slides next to highlight collagenous structures as red and background tissue as yellow and was left in situ for 30minutes. Dehydration was then performed using serial immersion of the slides in 100% ethanol and finally xylene. The slides were then mounted using DPX and analysed for collagen content (red) located around the periphery of muscle fibres, arteries and basal lamina. The slides were blinded at this point by assignation of a letter. Slides were examined under a light microscope and pictures taken with Zeiss Axioskop 2 mot (Carl Zeiss, Gottingen, Germany) (182;183).

3.4 Immunohistochemical staining of skeletal muscle to analyse fibre type specific morphometry

Standard immunohistochemistry using the avidin-biotin-complex (ABC) technique was used to detect and localise relevant antigens in skeletal muscle sections. The ABC technique involves three components. The first is the unlabelled primary antibody. The second is a biotinylated secondary antibody and the third layer is a complex of avidin-biotin peroxidase. The peroxidase is then developed with 3,3'-diaminobenzidine (DAB) (Vector Laboratories, UK), to produce a stable, brown chromagen.

3.4.1 Sequence of immunohistochemical staining for fibre typing

An initial section of a paraffin prepared block was checked for correct orientation using a routine normal haematoxylin and eosin stain. The block was blinded and assigned a letter. Once orientation was confirmed through Haematoxylin and Eosin examination, fifteen sequential sections of 3μm thickness were sliced using a microtome. These slices were placed upon polylysine treated slides (VW International UK) which were marked in series and according to the letter used for the blinded sample they were cut from.

3.4.2 Pre treatments used for antigen retrieval

There are two common methods for antigen retrieval:

- Heat Induced Epitope Retrieval (HIER) to destroy the cross links formed by formalin immersion in a retrieval solution such as TRIS-EDTA (pH=9.0) or Citrate (pH=6.0) In this study, TRIS-EDTA was used commonly and samples were heated for 20 minutes on full power in the microwave

- Proteolytic Induced Epitope Retrieval (PIER) which through enzyme digestion with enymes such as proteinase k, trypsin, chymotrypsin and pepsin makes antigenic sites available for detection.

Serial sections of 3μm thick paraffin embedded muscle were immersed in xylene through ethanol to dewax and rehydrate the muscle sections. The samples were then placed in 590mls of methanol with 10mls of 30% Hydrogen peroxide to eradicate endogenous peroxidase action. After 10 minutes, the samples were rinsed in PBS and heated in a microwave on full power for 20 minutes in pre warmed TRIS/EDTA buffer, pH=9.0 for the purposes of antigen retrieval (78;184). This was made up from 12gms TRIS, 1 gm EDTA and the slow addition of 1M HCl until monitoring with a pH meter confirmed the solution had a pH=9.0. The buffer was then made up to 500mls with deionised water. The whole solution was further diluted x10.

For some optimisation experiments only, a citrate buffer pH=6.0 was used for pre-treatment instead of EDTA and was made up as follows: 2.1gms of Citric acid monohydrate was added

to 950mls of deionised water. 12.5mls of 2M Na OH was later added to this solution with the pH of the buffer continually monitored and finally made up to a litre with deionised water. In pre treatments with the citrate buffer, sections were placed in a microwave at full power for 7 minutes.

3.4.3 Incubation Conditions of Primary Antibodies

After removal from the TRIS/EDTA buffer, the slides were kept well hydrated and using a hydrophobic PAP pen, the sections were circled and 10% Normal Goat Serum (NGS) was applied. This reduced the amount of non-specific staining present in the tissue and chosen due to the species that the secondary antibody was raised in. The 10% NGS was left in situ for 20 minutes before being aspirated. The primary antibodies were applied to sequential slides after optimisation of dilution and incubation conditions had been established for each MHC antibody. These are laid out in Table 6.

3.4.4 Negative Controls

Negative controls were applied by either omitting the primary antibody and using a similar volume of PBS but performing the described protocols as outlined previously (179) or by exchanging the primary antibody with isotype matched control antibodies, i.e.serum immunoglobulins of the species from which the primary antibody was raised. Relevant dilutions were performed to ensure comparable concentration of primary antibody used.

3.4.5 Secondary Incubation and DAB Development

After the appropriate incubation time, (**see Table 6)** the slides were washed three times for ten minutes in PBS and biotinylated secondary antibodies (all goat anti-mouse species, 1:200 Vector Laboratories) were applied for 30 minutes. At the same time, the Avidin-Biotin Complex (ABC) was made up and left for 30 minutes at room temperature. Following three rinses in PBS for 10 minutes, the slides were incubated in the ABC complex for a further 30 minutes.

A further wash and finally 3, 3'-diaminobenzidine tetrahydrochloride (DAB) as the visible stain was applied for ten minutes.

The slides were washed yet again in PBS and for the purpose of counter staining nuclei, immersed in Mayer's Haematoxylin for 50 seconds, then running water, followed by a long dip in acid alcohol, running water and finally 5 long dips in blueing solution before a final long wash and dehydration through ethanol and xylene. DPX was used as a mounting medium.

Table 6: Optimisation of MHC Antibody Expression.					
MHC Stained	**Clone Number**	**Supplier**	**Species Type**	**Optimised Concentration**	**Optimised Incubation Conditions**
Type I and I/IIa	M8421	Sigma Aldrich	Ms monoclonal IgG	1:4000	RT 1hr
Type IIa, IIx, IIa/x	A4.74	Alexis Biochemicals	Ms monoclonal IgG	1:50	4°C ON
Type IIa, IIx, IIa/x	sc71	Professor J. Morgan, MRC, Imperial College	Ms monoclonal IgG	1:20	4°C ON
Neonatal	NCL-MHCn	Novacastra Laboratories	Ms monoclonal IgG	Variable	Variable
Developmental	NCL-MHCd	Novacastra Laboratories	Ms monoclonal IgG	Variable	Variable
All MHCs Except IIx	BF-35	Professor J. Morgan, MRC, Imperial College	Ms monoclonal IgG	Variable	Variable

Table 6: All slides were pre treated for antigen retrieval with heat and either citrate buffer or EDTA buffers as above. Optimal expression was determined by adjusting the concentrations of primary MHC antibodies and incubation conditions which vary by incubation time (1 hour to Overnight [ON]) or temperature (4°C to Room Temperature [RT]):

3.5 Sc71:

This was used to corroborate the staining of A4.74 and to ascertain whether the reported weak staining of IIx fibres was legitimate (175;185). A third sequential slide after fast and slow Myosin Heavy Chain antibodies was utilised and the same 100 fibres previously recorded were identified and examined again. It has been previously reported to stain IIa strongly and IIx weakly unlike other fast myosin antibodies, but like other fast myosin antibodies has no way of distinguishing between IIa and the IIa/x hybrid (57).

3.6 Optimisation of BF-35, Neonatal and Embryonic Myosin Antibodies

Although antibody 6H1 has been reported to stain type IIx fibres in frozen mammalian muscle sections there is currently no individual antibody to positively identify type IIx fibres nor the IIa/x hybrid in paraffin embedded adult human skeletal tissue. An attempt was made to identify IIx fibres through use of the "negative" stain with BF-35 (kindly donated by Professor Jenny Morgan, MRC Imperial College) although all prior experiments reporting this antibody as reliably staining all Myosin Heavy Chains except IIx had only been reported in mouse or rat frozen sections only (57). Optimisation was attempted in paraffin embedded sections.

Concurrently, muscle sections were also subjected to neonatal and embryonic (NHC-emb and NHC-n: Novocastra Laboratories, UK) myosin antibodies to determine whether either of these two phenotypes could be detected in adult skeletal muscle, particularly in view of the presence of some indeterminable fibres as well as the expected increase in disordered

myogenesis. Different pre-treatments for antigen retrieval, EDTA pH=9, citrate pH=6, with and without protein kinase, as well as altering incubation conditions such as length of time of exposure to the primary antibody, were carried out in order to show positive chromagen expression.

3.7 CD31 Endothelial Marker

A marker of all vascular endothelium, CD31 was used to compare vessel density in the two patient groups. The next sequential 3μm slice after the fibre type antibodies was exposed to the same pre-treatment protocol as before EDTA and heated in a microwave for 20 minutes, blocked with 10% NGS at room temperature and CD31 antibody (Dako M823), diluted to a concentration of 1:100 in Antigen Target Retrieval Solution (Dako) and left in situ on the slides for one hour. The rest of the ABC protocol described earlier was followed. After development of DAB for 10 minutes, the slides were counterstained with haematoxylin, dehydrated and preserved with DPX. Slides were examined under a light microscope and pictures taken with Zeiss Axioskop 2 mot (Carl Zeiss, Gottingen, Germany).

3.8 Satellite Cell Markers

3.8.1 Satellite cell markers Sequence of Staining

Sequential sections of muscle samples already analysed for fibre type and endothelial cells were further analysed for satellite cell markers due to the increased fibre flux and hybrid types seen in CLI. An investigation into satellite cells responsible for the increased but disordered myofibre regeneration seen in morphometric, light microscopy and TEM examination of ischaemic muscle may help elucidate the failure of the myogenic plastic response or suggest a source of stem cells that could act as a source of new muscle, adequate and maturing angiogenesis or neural repair.

Sections were stained in the sequence following CD31, CD34, pax7 and finally MyoD and the same five randomly generated fields were analysed as Figure 28 illustrates.

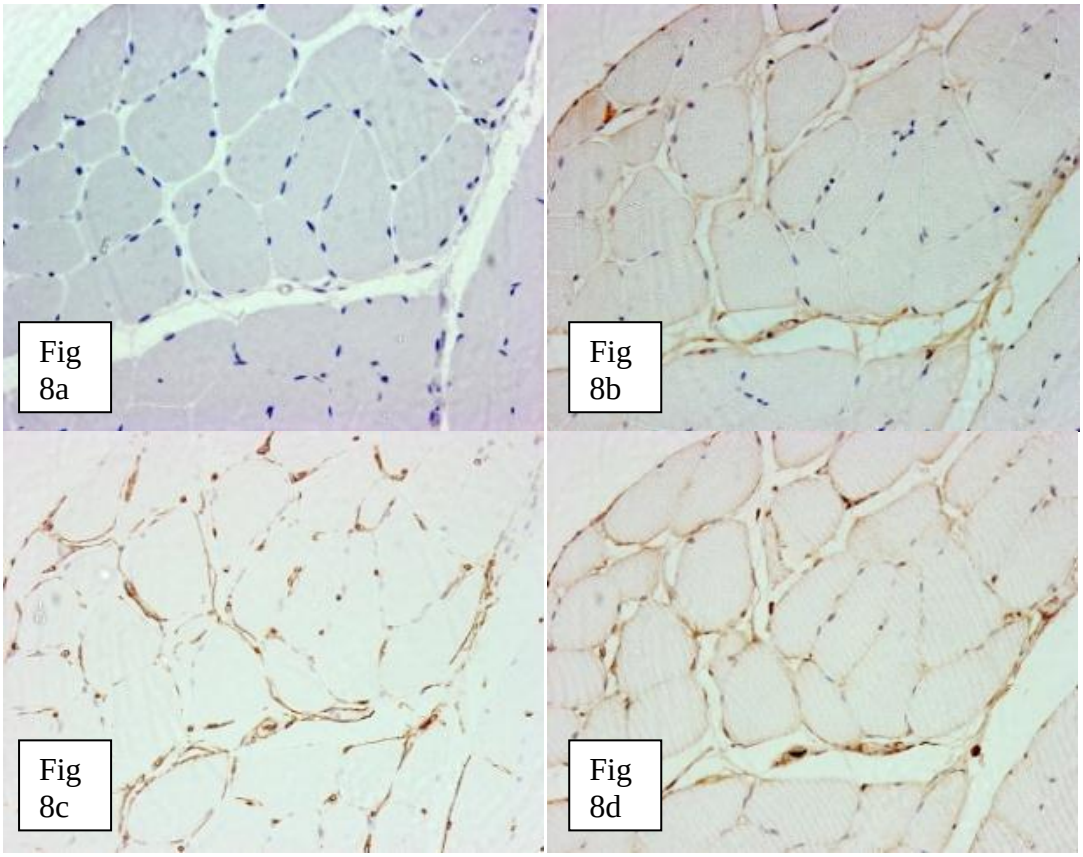


Figure 8a-d: Sequence of Staining of Satellite Cells: of Patient A (Control Group) illustrating sequence of antibodies raised against the following antigens in each patient. 8a: CD31 Included to show continuity of sequential slides. 8b:CD34, a marker of haematopoietic stem cells and quiescent satellite cells was included to quantify the presence of these cells (186) 8c:pax7 a marker of satellite cells 8d: myoD, a marker of differentiation, myoblast activity and active satellite cells.

3.8.2 Immunohistochemical recognition of satellite cell markers on sequential slides

The same five fields used in previous analysis for Picrosirius Red, MHC isoforms and CD31 expression were identified and further analysed in sequential blinded slides using pax7, CD34 and MyoD antibodies as outlined above. The same method of ABC and DAB chromagen

staining was utilised as described in **Section 3.4.2** and optimisation of the antibodies is displayed in **Table 7.**

Table 7: Antibodies used for tissue analysis and satellite cell recognition

Optimal expression was determined by adjusting the concentrations of primary MHC antibodies and incubation conditions. Incubation Conditions Vary by Incubation time (1 hour to Overnight [ON]) or Temperature (4^{o}C to Room Temperature [RT]):

Ab	Clone	Source	Species	Conc.	Pre-Treatment	Incubation
CD34	QBEnd-10	Dako	Mouse monoclonal IgG	1:50	EDTA 20m	RT 1 hour
Pax7	QC2152	Aviva Systems	Rabbit Polyclonal	1:200	No PT	4^{o}C ON
MyoD	5.8A	Dako	Mouse monoclonal IgG	1:50	EDTA 20m	4^{o}C ON

Pre blinded slides were analysed using the Zeiss Axiosopp 2 light microscope at x200 magnification coupled with an Olympus camera. Image capture was performed and JPEGs generated were stored to be analysed. Positive stains for each antibody were recorded in five previously randomly generated fields per patient sample and subjected to data analysis as outlined later. The number of cells or nuclei staining positively for each field per sample was recorded in the sequential areas of assessable muscle per patient biopsy and a comparison was made in each group using the Mann Whitney U Test.

3.9 Data Collection

3.9.1 Orientation of Samples

Skeletal muscle sections were first stained with a simple H&E stain to determine correct orientation of the myofibres in each sample. It was ensured that all samples had fibres presented perpendicular to the microtome with the resulting tissue presented in cross section as shown below in Figure 9.

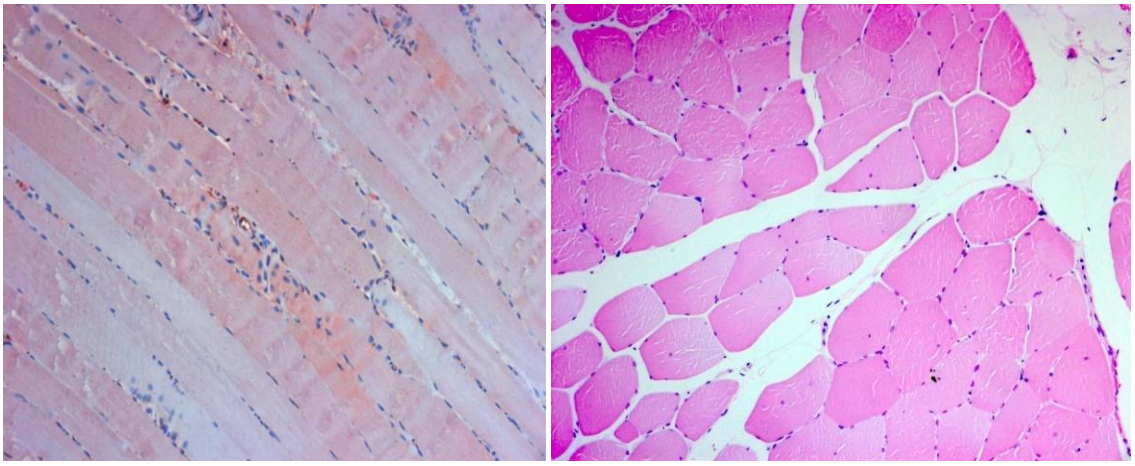


Figure 9: Orientation of Samples x200 magnification. 9a: shows longitudinal preparation of the tissue for TGF-β antibody. Z lines are apparent; nuclei are generally located in lines parallel to the myofibres and at junctions. 9b illustrates the correct plane suitable for morphometry, perpendicular to the myofibres as described previously (176;181;187;188).

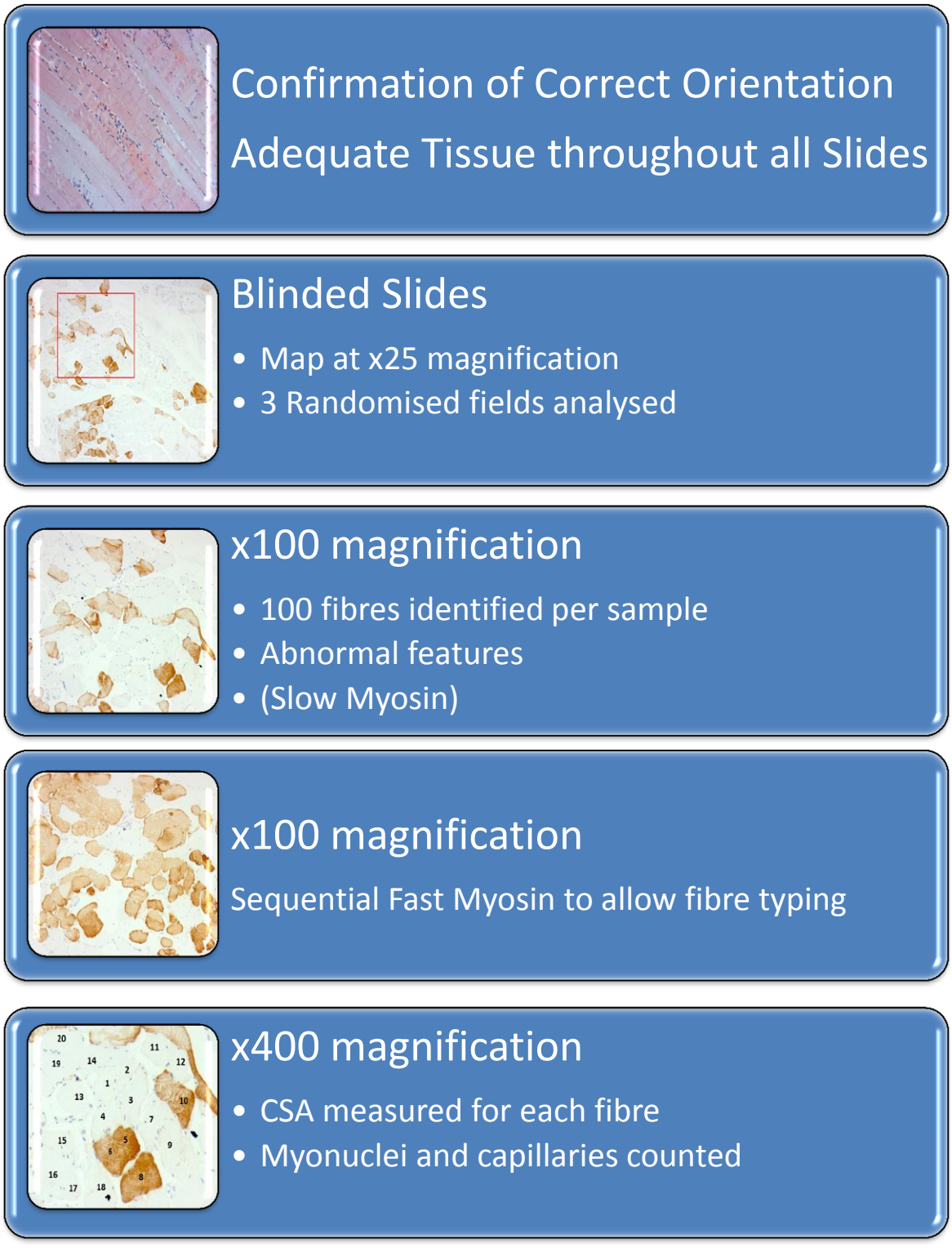


Figure 10: Methods of Morphometric Data Collection

3.9.2 Blinding of Samples

All samples were blinded and assigned a unique coded identifier. This was performed by an independent observer who retained the identity of the samples until after the analysis. This reduced bias in data collection.

3.9.3 Randomisation

Randomisation was achieved through the use of www.random.org an online random number generator. Each section was divided into 9 fields and examined based on the result of the random generator.

3.9.4 Morphometric Analysis

Slides were analysed using the Zeiss Axioskop 2 MOT microscope (Carl Zeiss, Gottingen, Germany) and the computer operating system used for analysis of slides was the Axiovision 4 system. 100 random fibres were identified at x100 magnification in each sample and numbered as previously described (175;180;180;181;189-191). The total number of fibres per five fields at x100 magnification was also noted.

As described by Gosker, only fibres without artefacts that were part of a cluster of ≥30 fibres were included (181). Each fibre was marked and recorded from 1-100, on a "map" at x2.5 magnification. Using the semi automated spline measuring function of Axiovision v4, both

the features and cross sectional area (CSA) of each fibre was noted alongside its number. This semi-automation as previously described allowed for the rejection from the study of those fibres affected by artefact (192;193).

The cross sectional area of each of these fibres was then measured at x400 magnification using the Axiovision v.4 computer analysis software program and the result recorded in µms. Care was taken to measure the inner surface of the basal lamina to avoid including thickened or collagenous tissue associated with muscle damage. The following were also recorded:

- Myonuclei/ fibre
- Fibres/ field
- Central nuclei
- Angular fibres
- Fibre type
- CSA (µms)

3.9.5 Fibre Typing With Sequential anti-MHCs

Blinded 3µm thick sequential sections were stained for type I/IIa and type II myosin heavy chains with M8421 and A4.74 antibodies respectively (175;176;194). Three different randomised fields per patient biopsy, containing a total of 100 fibres were analysed as per previously described protocols (180;181). Positively staining myofibres for each antibody raised against a fast or slow myosin were recorded (191-193;195) as positive or negative cytoplasmic brown DAB stain was anticipated to be apparent for each phenotype.

Slides were examined under a light microscope and pictures taken with Zeiss Axioskop 2 mot (Carl Zeiss, Gottingen, Germany). The same one hundred fibres identified and recorded above were examined and fibre typed according to the positive brown DAB stain exhibited in none, one or both sequential slides. A note was also made of the intensity of staining for each antibody. The first slide in the sequence was stained using the Avidin-Biotin Complex method to recognise those fibres containing type I (or slow) MHC. The M8421 (Sigma-Aldrich) clone also recognises those fibres containing type I/IIa (or hybrid type IIc) MHCs. A positive result was visualised through the brown DAB chromagen in the sarcoplasm. This was found to be very specific with little background signal. It was followed by the anti-fast MHCs A4.74 and sc71 according to previous optimisation. Neither anti-fast MHC labelled type I MHC. Whilst the A4.74 antibody was specific and displayed little background expression, it also appeared to differentiate between type IIa and type IIx fibres, with a stronger chromagen signal (dark brown) displayed from the former and a weaker signal displayed from the IIx fibres. No distinction could be made regarding type IIa/x hybrid fibres.

Optimisation of BF-35(all MHCs but IIx), neonatal and embryonic myosin in paraffin embedded tissue with attempts to change pre-treatment, concentration of antibody and variation in incubation times. No staining was achieved under any conditions.

Table 8 :MHC Isoform Identification			
Chromagen Expression In Sequential Slides		MHC Isoform Expressed	Corresponding Fibre (As Labelled Below)
Slow (M8421)	Fast (A4.74)		
++	-	I	C
++	++	I/IIa	A
-	++	IIa	B
-	++	IIa/x	B
-	Weak	IIx	D

Table 8: Identification of Myosin Heavy Chains Using Sequential Slides Stained with Fast (A4.74) and Slow (M8421) anti-Myosin Heavy Chain Antibodies and the Fibre Type generated by the two results as shown in **Figures 11i and 11ii** with a corresponding letter to indicate MHC isoform identified:

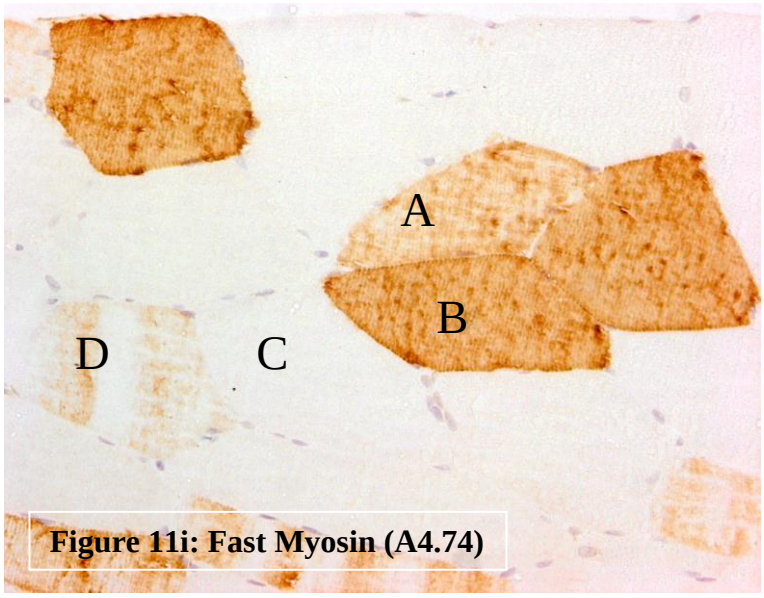


Figure 11i: Fast Myosin (A4.74)

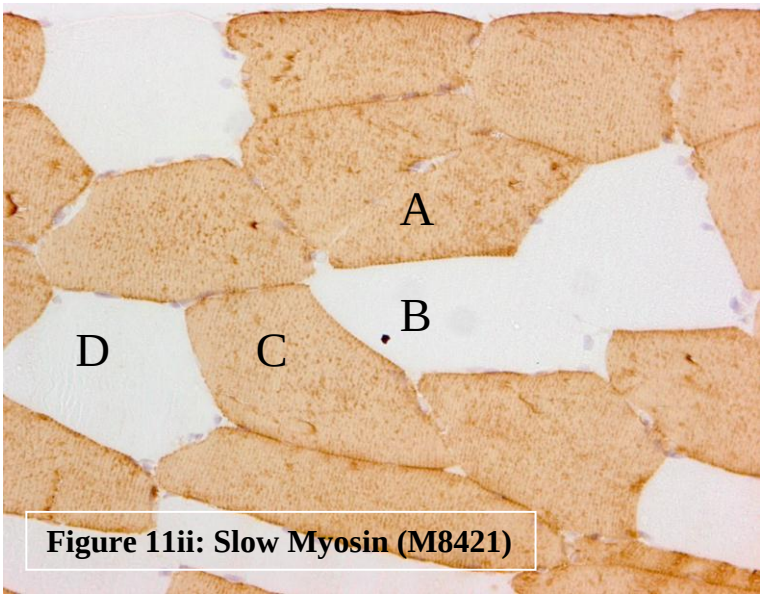


Figure 11ii: Slow Myosin (M8421)

Figure 11: Reactivity of skeletal muscle fibres in human gastrocnemius muscle with monoclonal antibodies directed against MHC isoforms. Three randomly generated areas in sequential slides were stained for Fast (i) and Slow (ii) Myosin Heavy Chain (MHC) isoforms. Corresponding fibres in sequential slides were identified and following established protocols (57;177;185;196) were labelled according to specific chromagen expression and marked using Table10 above as: A: IIc if there was expression demonstrated with both antibodies. B: IIa or IIa/x if there was expression only in the Fast Myosin slide. C: Type I if only Slow Myosin was expressed D: IIx if there was weak expression of Fast Myosin only.

3.9.6 Characterisation of active and quiescent satellite cells

In order to assess the number and activity of satellite cells in the two sample groups, frequency of pax7 antibody positive nuclei were evaluated to determine the overall number of satellite cells present. CD34 was utilised as a marker of haematopoietic stem cells. When pax7 was found to be co expressed with CD34 in sequential slides but not MyoD, these cells were identified as quiescent satellite cells.

Those cells co expressing pax7 and MyoD on sequential slides were identified as differentiating (or active) satellite cells. Other cells present in the tissue undergoing differentiation were myoblasts and a sarcoplasmic pattern of labelling was seen. The numbers of each group were assessed for five fields per sample and compared using the Mann Whitney U Test.

3.10 Protein Analysis of Satellite Cell Markers via Western Blot

Protein expression was quantified was using Western blotting with isoenzyme specific antibodies. Detection is based on antigenic molecular weight and interaction of the primary antibody with the antigen. The process of gel electrophoresis employs polyacrylamide gels and buffers loaded with sodium dodecyl sulfate (SDS).

SDS-PAGE (SDS- polyacrylamide gel electrophoresis) allows separation of proteins by molecular weight, maintaining polypeptides in a denatured state once they have been treated with reducing agents to remove secondary and tertiary structures. SDS confers a negative charge to the sampled proteins which then migrate to the positively charged electrode through the polyacrylamide structure of the gel. Smaller proteins migrate faster and the proteins fan out according to size. The higher the concentration of acrylamide, the better the resolution of lower molecular weight proteins as the pore size in the gel decreases. A lower acrylamide concentration allows better resolution of higher molecular weight proteins due to a larger pore size.

3.10.1 Preparation of Skeletal Muscle Tissue for Protein Analysis

Muscle biopsies measuring $5mm^3$ were immediately snap frozen in liquid nitrogen after harvesting and stored at -80ºC until further use.

3.10.2 Homogenisation of Skeletal Muscle

Protein expression of pax7 in both sample groups was quantified using the Western blot technique with the aforementioned specific primary antibodies probing for their particular antigens and detection via densitometry of the ensuing chemiluminescence was used to quantify the difference in protein levels between the two groups.

All muscle samples were homogenised in short bursts to avoid heat damage to the proteins, by an electric homogeniser, (Ultra-Turrax T8, IKA Werke GmbH & Co. KG, Staufen, Germany).with the samples immersed in ice at all times. This was performed using a homogenising buffer which was made up from Tris/HCl, (25mM at pH=7.4) EDTA (1mM) and EGTA (1mM). 50µls was added to each sample. This was then centrifuged at 13,000 rpm for 10 minutes as per previously described experiments (148). The supernatant was drawn off and added to 20µls of x4 Laemelli or loading buffer (20% sodium dodecyl Sulphate, 1M Tris/HCl pH6.8, glycerol, mercaptoethanol, 0.2% bromophenol blue and deionised water) and added to a water bath at 90˚C for 5 minutes. 10µls of a molecular weight marking ladder to confirm the molecular weight of probed bands was then loaded into the first well of a pre prepared 4%:20% Tris glycine polyacrylamide gel.

3.10.3 Electrophoresis

A running buffer of PBS Tween, which allows for a constant current, was then added and a current of 125mV was applied across the polyacrylamide gel for 90 minutes to separate the proteins within the lysate according to their molecular weight, the smaller molecules moving

further across the gel. Once the visible Laemelli buffer was visualised at the bottom of the wells, the current was switched off.

3.10.4 Electrotransfer

The cassette containing the gel was opened, the latter transferred onto a nitrocellulose Hybond C-Extra membrane (Amersham Biosciences UK) and between a sandwich of blotting paper and bubble free sponges in a transfer buffer made up of 50mls methanol, 20mls 25x Tris Glycine Buffer, made up to 500mls with deionised water. Using transfer apparatus, 35mV were applied across the sandwich for 1.5hours and in this way, proteins already separated by electrophoresis were transferred from the polyacrylamide gel to the Hybond C-Extra membrane.

3.10.5 Probing With Primary Antibodies

The membrane was then blocked with 2.5gms of milk to 50mls PBS/Tween for an hour to eliminate any non-specific binding of primary antibodies. The primary antibody (pax7 1:2000) was diluted in PBS/Tween then applied to the membrane overnight at 4ºC. After three further rinses with 0.05% PBS/Tween for half an hour, the appropriate secondary horseradish peroxidase-conjugated affinity purified goat anti-rabbit (pax7) or anti-mouse (CD34/MyoD) secondary antibody (Vector Laboratories) was added to the membrane at room temperature for an hour at the concentration of 1:3000 in each case before being washed finally in PBS/Tween for another half hour to avoid background chemiluminescence.

3.10.6 Development and Densitometry of Detected Bands

The membranes were finely covered with a luminol chemiluminescence detection reagent (Enhanced Chemiluminescence ECL Kit, Amersham Biosciences) and the membranes were wrapped in cling film and finally transferred in a dark room onto ECL Hyperfilm inside an X-ray cassette case for an optimal time (between 30 seconds and 5 minutes) to gain optimal bands with minimal overexposure from the chemiluminescent signal. Once achieved, the Hyperfilm was washed in water, fixative and finally developing fluid before being left to air dry.

Due to the poor or non-specific signalling of CD34 and myoD antibodies, the above method used for pax7 was modified as follows. The membrane was blocked in 5% bovine serum albumin (BSA) for an hour before the primary antibodies CD34 or myoD were placed on the membrane and these were incubated at the following concentrations overnight at 4°C: 2.5µls CD34 in 5mls of 1x TRIS buffered saline (TBS) [1:2000]; 5µls of MyoD in 1x TBS [1:1000]. The membrane was then thoroughly rinsed three times for 10 minutes in TBS. The secondary antibody (HRP-conjugated goat anti mouse) was applied at 1:2000 dilution for an hour. This was washed off in TBS three times over half an hour and developed as outlined above.

3.10.7 Internal Control of Protein Concentration

To confirm equal loading between samples, membranes were stripped by washing in PBS-Tween. Mouse monoclonal primary antibody GAPDH (Abcam 1:10,000) was applied to the membrane for an hour, followed by a goat anti- mouse HRP linked secondary antibody

(1:10,000), following the same steps outlined above. The western blot images were analysed and quantified by densitometry using Biospectrum® Imaging System and VisionWorks®LS Software (UVP). Quantification of the probed antigens was expressed in relative density units (RDU) according to the strength of the luminescence.

3.11 Statistical Analysis of Results

Patient demographics were compared using Fisher's Exact Test if $n<5$ or χ^2 if $n>5$. χ^2 is useful to analyse binary or categorical data and compares associations between proportions. Continuous quantitative data were expressed as median values and, for non-parametric data and mean and standard deviations for parametric data from experiments using human skeletal muscles inter-quartile ranges were included in the appendices.

Single comparisons between values in the two patient groups for non parametric two independent samples were performed using two-tailed Mann-Whitney U test using Graph Pad Prism v4.0 software $p.<0.05$was taken to be statistical significance with $p.<0.0001$inferred as highly significant .

Chapter 4: Results

Qualitative description of ischaemic muscle morphology through the use of light and transmission electron microscopy:

4.1 Introduction

Ischaemic tissue displays characteristic and pathognomonic changes arising from local effects on myofibres or due to damage on their nerve supply. Atrophy has been reported as common, although there is no consensus as to which fibre types are more susceptible to damage. The deposition of fibrofatty tissue and disordered myogenesis has been reported before. There is little evidence regarding the number of myonuclei pee fibre in CLI nor regarding the numbers of regenerating, angular fibres nor hybrid fibres. The body of research into fibre type shift and plasticity under ischaemic conditions is contradictory in its findings.

In this present study, Transmission Electron Microscopy was first used to confirm that there is a change in skeletal muscle architecture. This was later confirmed by light microscopy and morphometric data was collected through the use of both histological and immunohistochemical means. This study aims to show the adaptive potential displayed by the human gastrocnemius muscle in response to chronic ischaemia, demonstrated by histochemical, immunohistochemical and morphometric means.

The aims of this study were as follows:

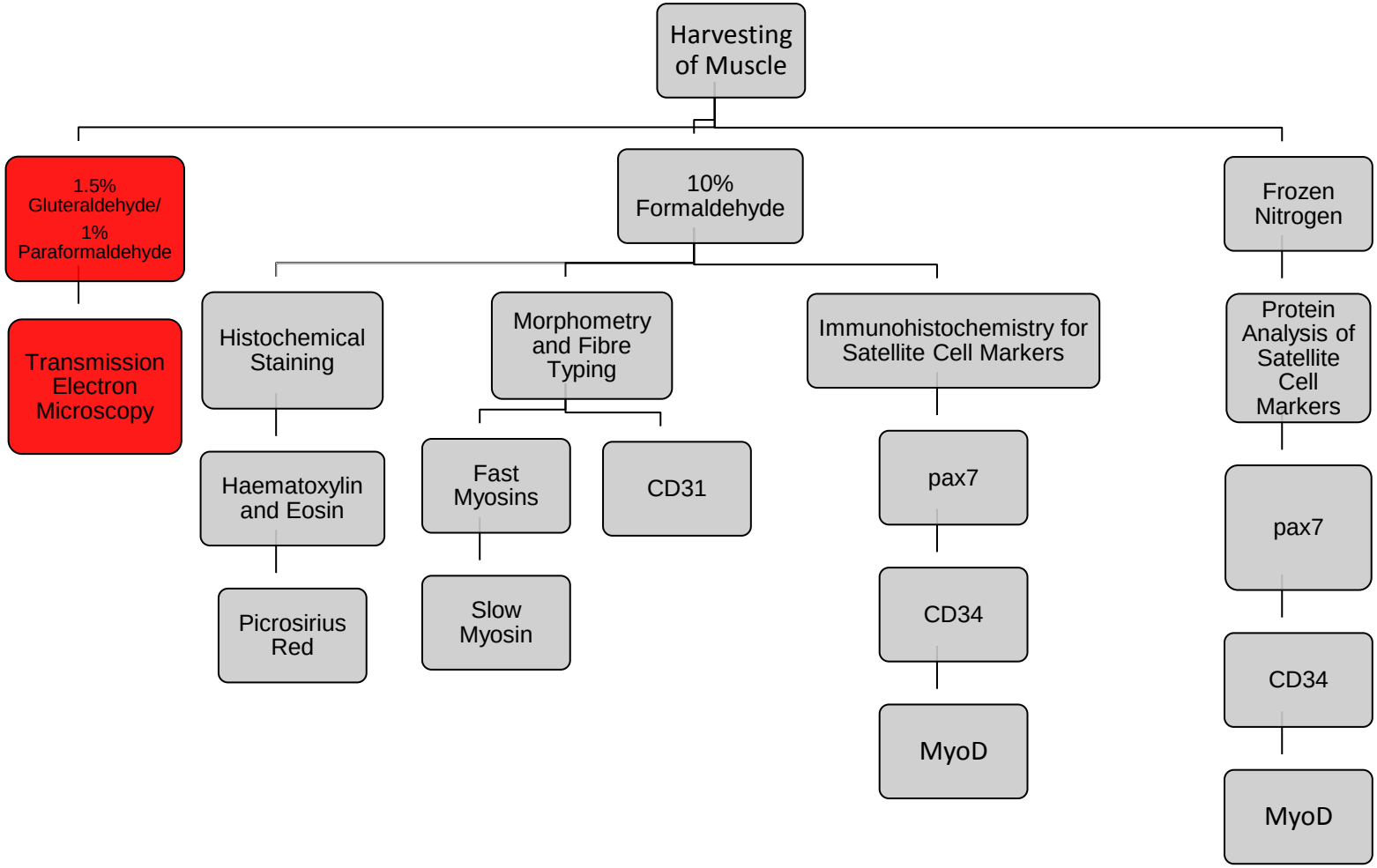
4) To describe the morphological changes exhibited by human skeletal muscle in response to critical limb ischaemia through the use of both light and transmission electron microscopy

5) To quantify those changes morphometrically.

6) To examine the adaptive mechanisms of skeletal muscle; in particular the response of resident stem cells to critical limb ischaemia.

In pursuing these questions, multiple modalities were undertaken. In particular, to assess the architectural changes seen in chronically ischaemic tissue, observations regarding the morphology of the tissue can be made through the use of both electron as well as light microscopy to assess the “end-organ”, or skeletal muscle.

Figure 12: Transmission Electron Microscopy of CLI Tissue



4.1.1 TEM Examination of Ischaemic Adult Human Skeletal Muscle Tissue Reveals Disorder in Ultrastructure Morphology

Muscle samples were harvested and prepared (as described in **Section 3.2**) after informed consent was gained., TEM was used to examine differences in the ultrastructure of ischaemic compared to non ischaemic samples.

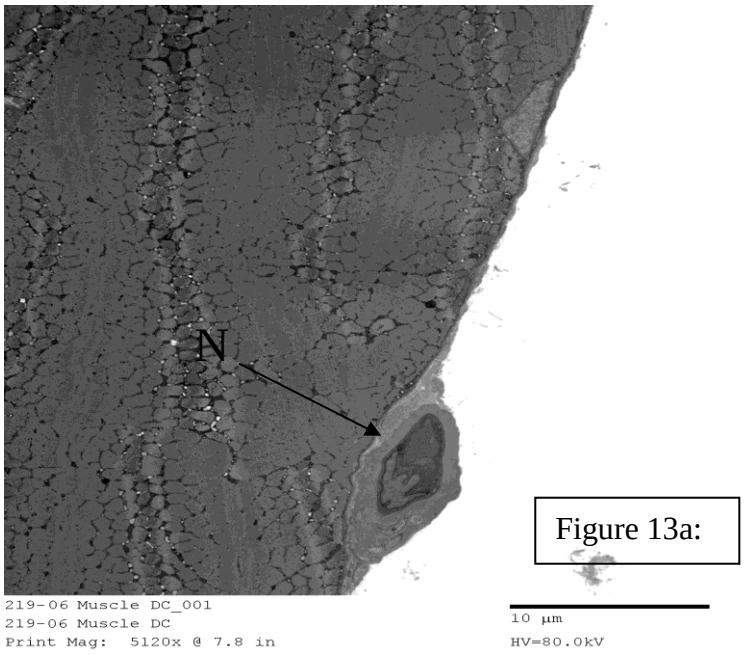


Figure 13a:

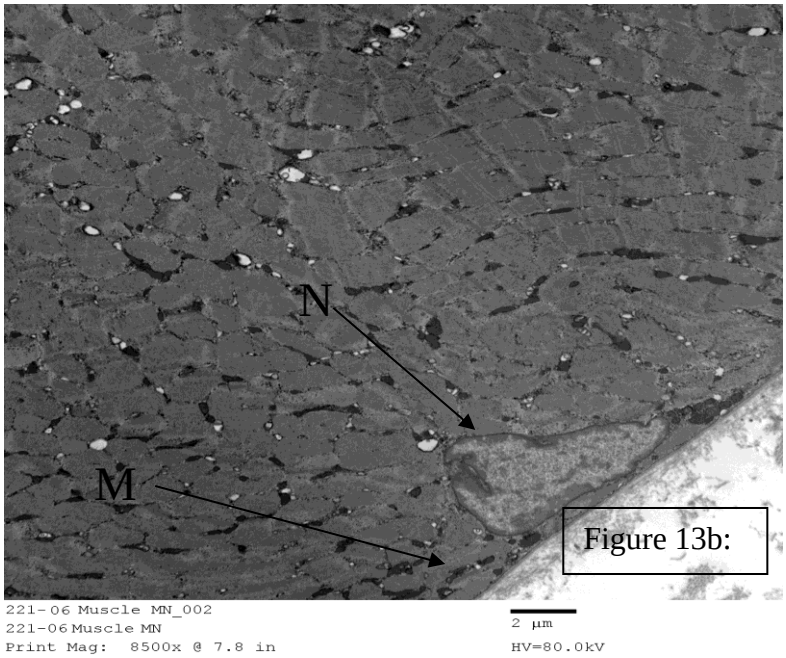
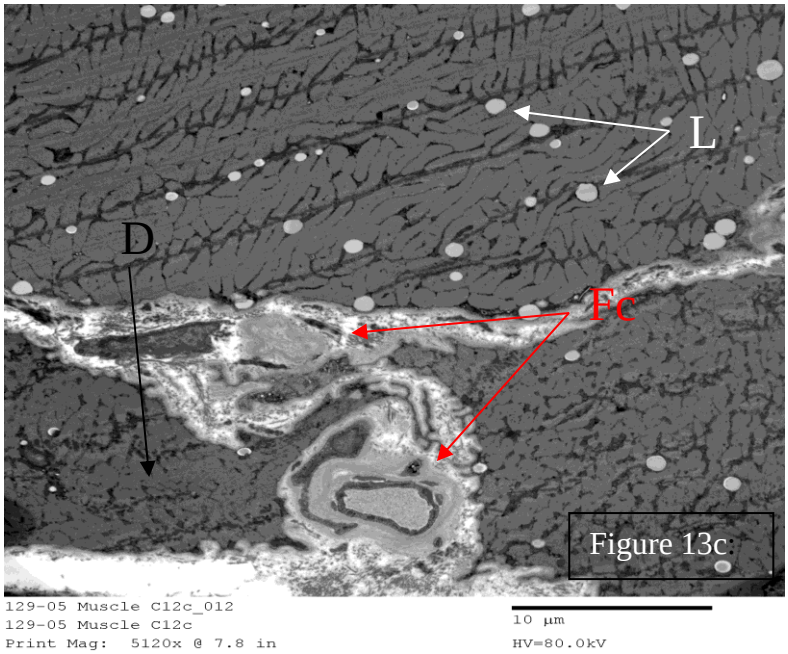


Figure 13b:



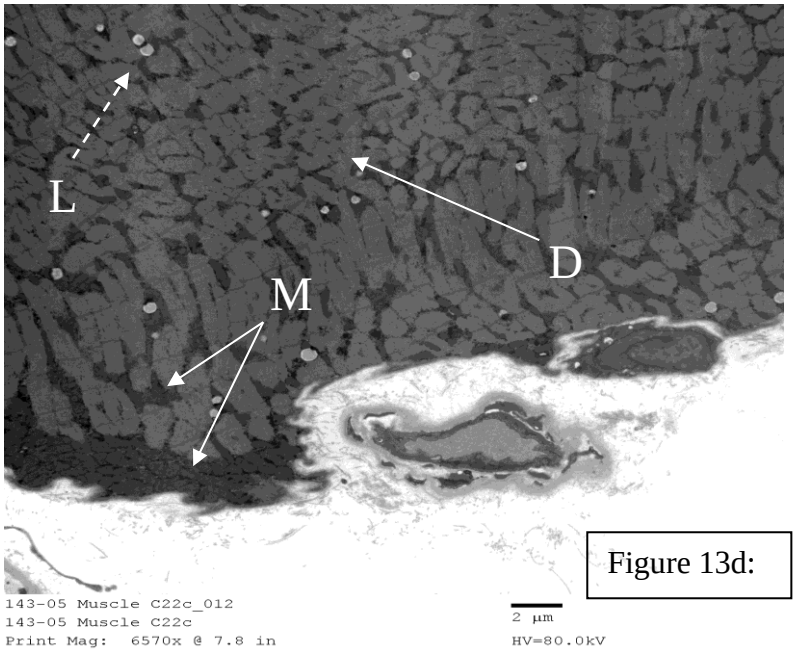


Figure 13: TEM examination of CLI Muscle 13a,b: x5120 and x8500 magnification. 13c,d: Ischaemic Muscle at x 5120 and x 6570. Nuclei in Control tissue were uniform and ovoid (N). Lipofuscin droplets (L) can be seen in ischaemic muscle with increased numbers of oedematous mitochondria (M), particularly towards the sarcolemma. Fibrocollagenous deposition is also observed particularly around capillaries (Fc). Disorganised myofilaments were seen in abundance (D).

Myofilaments were found to be highly disorganised in areas, consistent with the loss of normal polygonal architecture visualised in conjunction with the loss of orientation sometimes seen in light microscopy of ischaemic muscle. This may also account for some of the increased variability in fibre size. Two types of inclusion bodies of the sarcoplasmic reticulum were noted. The first were light, featureless and amorphous and the second type were dark aggregated structures with associated granules. Mitochondria, dark ovoid bodies, were seen to be swollen in ischaemia and occupied areas near satellite cells, the basal lamina and capillaries.

Ischaemic muscle examined under TEM reveals occasional internalised or central nuclei, also visible in light microscopy. There were also found to be numerous multilobed myonuclei indicating mitogenesis and often with thick nuclear membranes and less dense chromatin contained within, features not observed in control muscle. All the above features correlate well with previous findings (82;197;198).

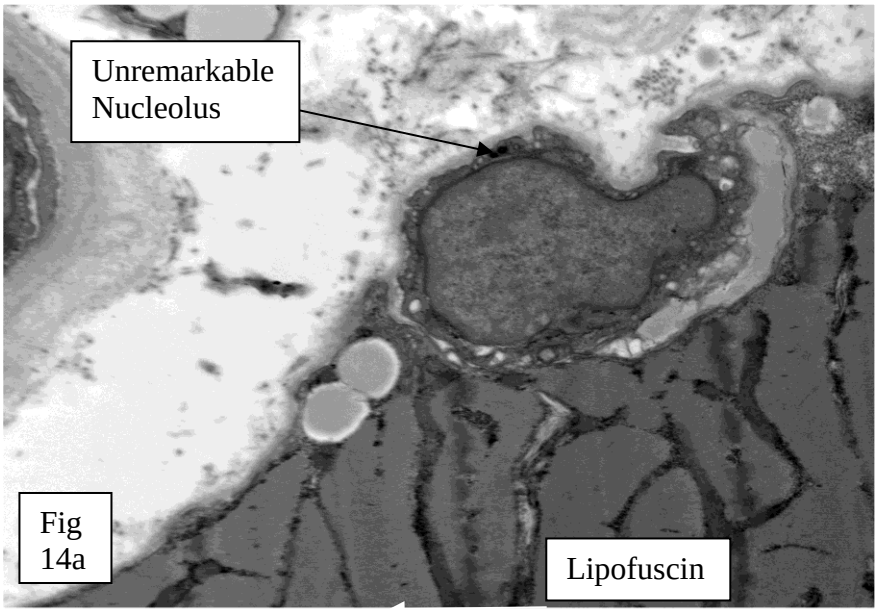
Lipid or lipofuscin droplets, by products of mitochondrial metabolism and lipid peroxidation (199-201), can be commonly seen as light circles resident between ischaemic myofilaments, and the increased fibrocollagenous structures around capillaries and between fascicles correlates well with the fibrofatty infiltration seen on light microscopy of similar tissue. Most ischaemic samples contained more mitochondria, seen as dark ovoid structures between fibres **(see Figure 13d)**

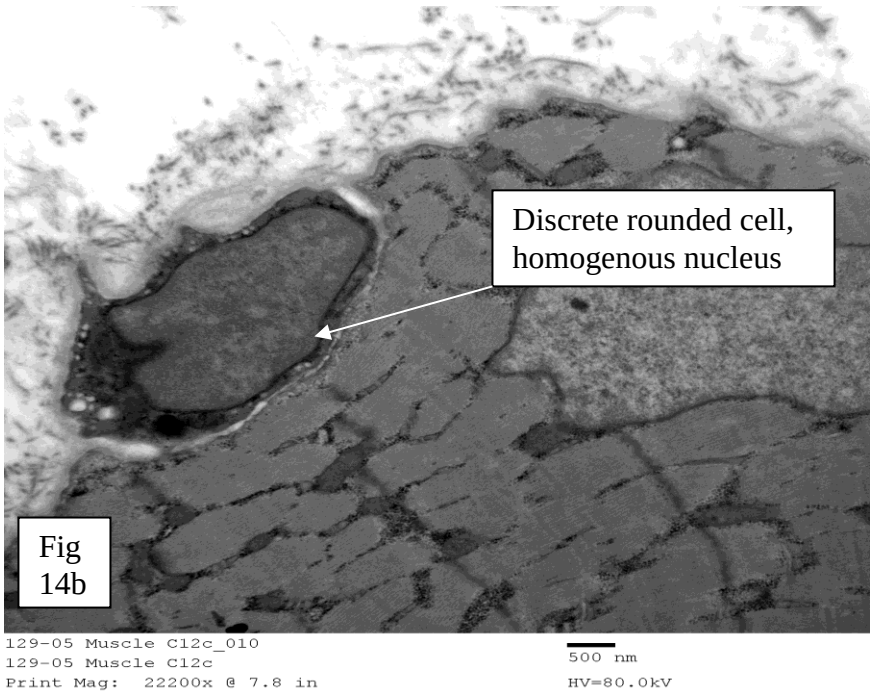
4.1.2 TEM Examination of Satellite Cell Characteristics in Ischaemic Tissue

Quiescent satellite cells are in the order of 6.5μms in length, 2.9μms in width and generally oval in shape. They were found to contain a large rounded/oval nucleus occupying approximately 60% of the total transverse area of each cell **(see Figure 14a)**. In primates, quiescent satellite cells are significantly smaller than those seen in ischaemia ($p < 0.01$), with a smaller percentage of nuclear to cellular area ($p < 0.01$) than active cells (82). The quiescent satellite cells were noted to have no projections and there was little electron-dense material present in the intercellular gap as described by Gregory and Mars (2004). Active cells are reported as larger than quiescent satellite cells with a more pronounced nucleus, often lobular in appearance. Unlike the quiescent cells, the active cell has projections via the basal lamina into the substance of the myofibre. They also display the well defined nucleolus noted also by Dedkov (124).

Transmission Electron Microscopy was used to determine whether there was any noticeable difference in the numbers of satellite cells observed in ischaemic compared to non-ischaemic muscle. To this end, tissue was prepared as described earlier **(section 3.2)** and examined with the TEM. Representative images are recorded below. Of note, an area of three ischaemic samples and five control samples, measuring approximately 1mm x 1mm was visualised.

It was observed that while there were abundant satellite cells noted throughout all the ischaemic samples, they were not seen in 4 out of 5 control samples and only occasional cells seen in one sample. This concurs with observations in the literature by previous groups that in normal muscle, satellite cells are rarely seen (202).


Unremarkable
Nucleolus
Fig
14a
Lipofuscin
129-05 Muscle C12c_011
129-05 Muscle C12c
Print Mag: 22200x @ 7.8 in
500 nm
HV=80.0kV


Discrete rounded cell,
homogenous nucleus
Fig
14b
129-05 Muscle C12c_010
129-05 Muscle C12c
Print Mag: 22200x @ 7.8 in
500 nm
HV=80.0kV

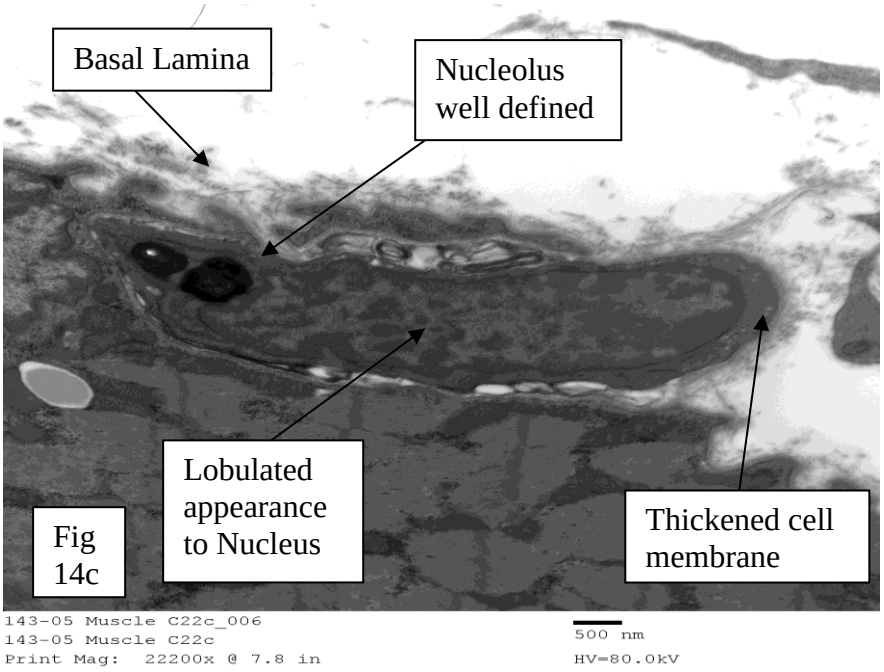
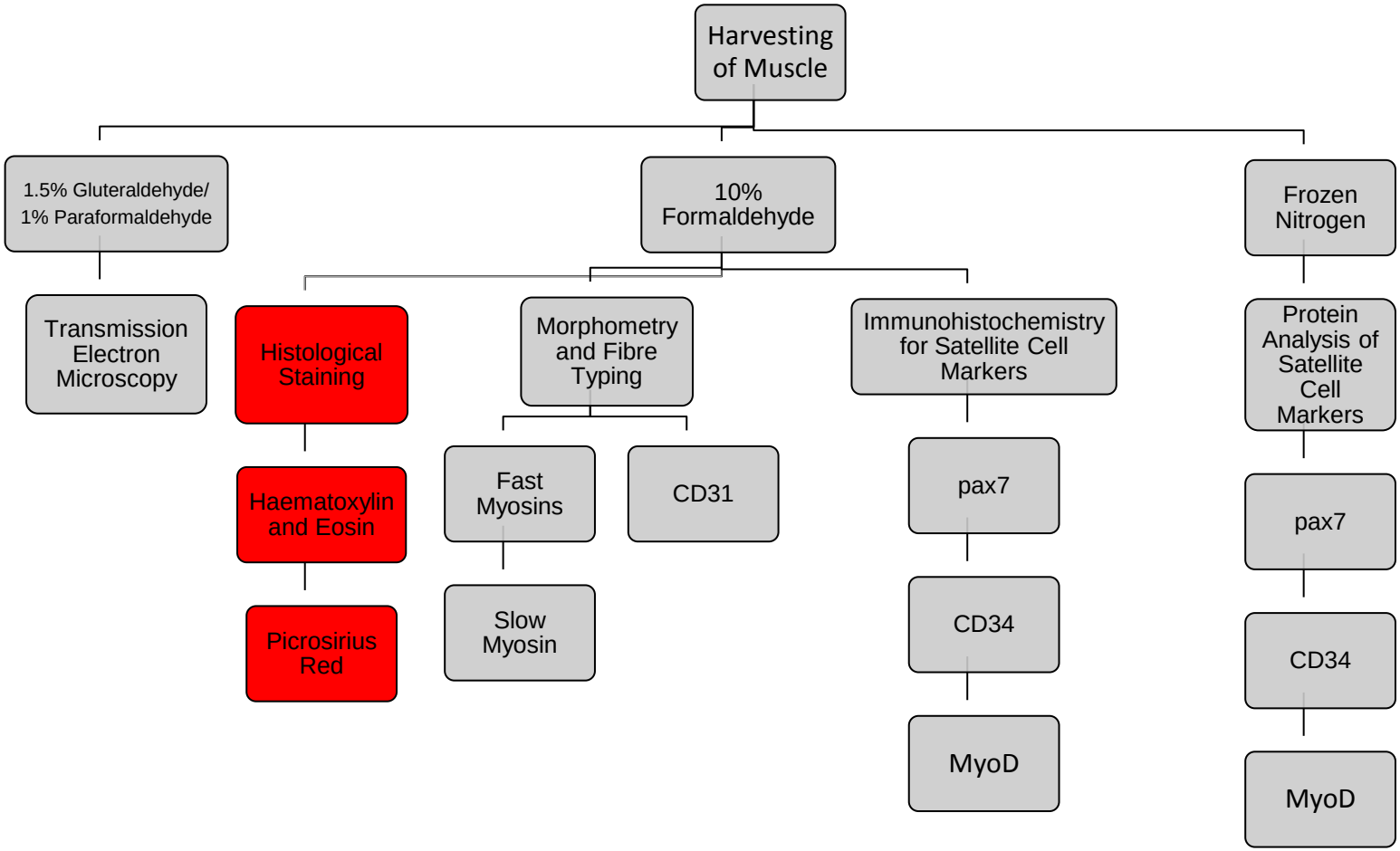


Figure 14a: TEM Quiescent Satellite Cell contains unremarkable organelles, has a smooth ovoid shape, well defined basal lamina with no projections **Figure 14b**: Mobilising satellite cell, with nucleus appearing to become bilobar. **Figure 14c**: Multiple mitochondria seen in satellite cell with enlarging nucleolus and lobular nucleus as previously described (82)

4.2 Light Microscopy

4.2.1 Figure 15: Histological Examination



4.2.2 Picrosirius red stained ischaemic adult human skeletal muscle confirms changes seen under TEM

The hypothesis that there is a change in skeletal muscle morphology and morphometry as a result of critical limb ischaemia led to the need for an overall analysis of harvested tissue through a non-immunologically based stain. Sequential sections were thus stained as described earlier with Picrosirius Red (**Section 3.3.1.2**) to observe this basic tissue morphology and morphometric changes visible in CLI skeletal muscle compared to control muscle from patients undergoing coronary artery bypass grafting.

Picrosirius Red staining was utilised primarily to determine the correct orientation of tissue, but also to determine some basic morphological data. It was chosen to demonstrate the increase in collagenous content in ischaemic tissue and over Masson's Trichrome due to the finding of some researchers that the latter tends to underestimate collagen deposition (183).

Nuclei were observed stained in black, cytoplasmic/ sarcoplasmic contents as yellow and collagen appeared red in colour. Myosin heavy chains have not been reported to differ with this stain and therefore morphometrics were carried out on these samples so bias was not introduced when measuring CSA.

The most striking aspect of the tissue is the loss of the normal tight polygonal architecture seen in normal skeletal muscle. 70% (n=7) of ischaemic samples displayed this loss, yet only 10% (n=1) of the control group did so. Fibres, in yellow, appear to be smaller and there is a much greater diversity in size and shape with cellular atrophy and regeneration occurring in greater number. This is similar to the effects seen in muscular dystrophy.

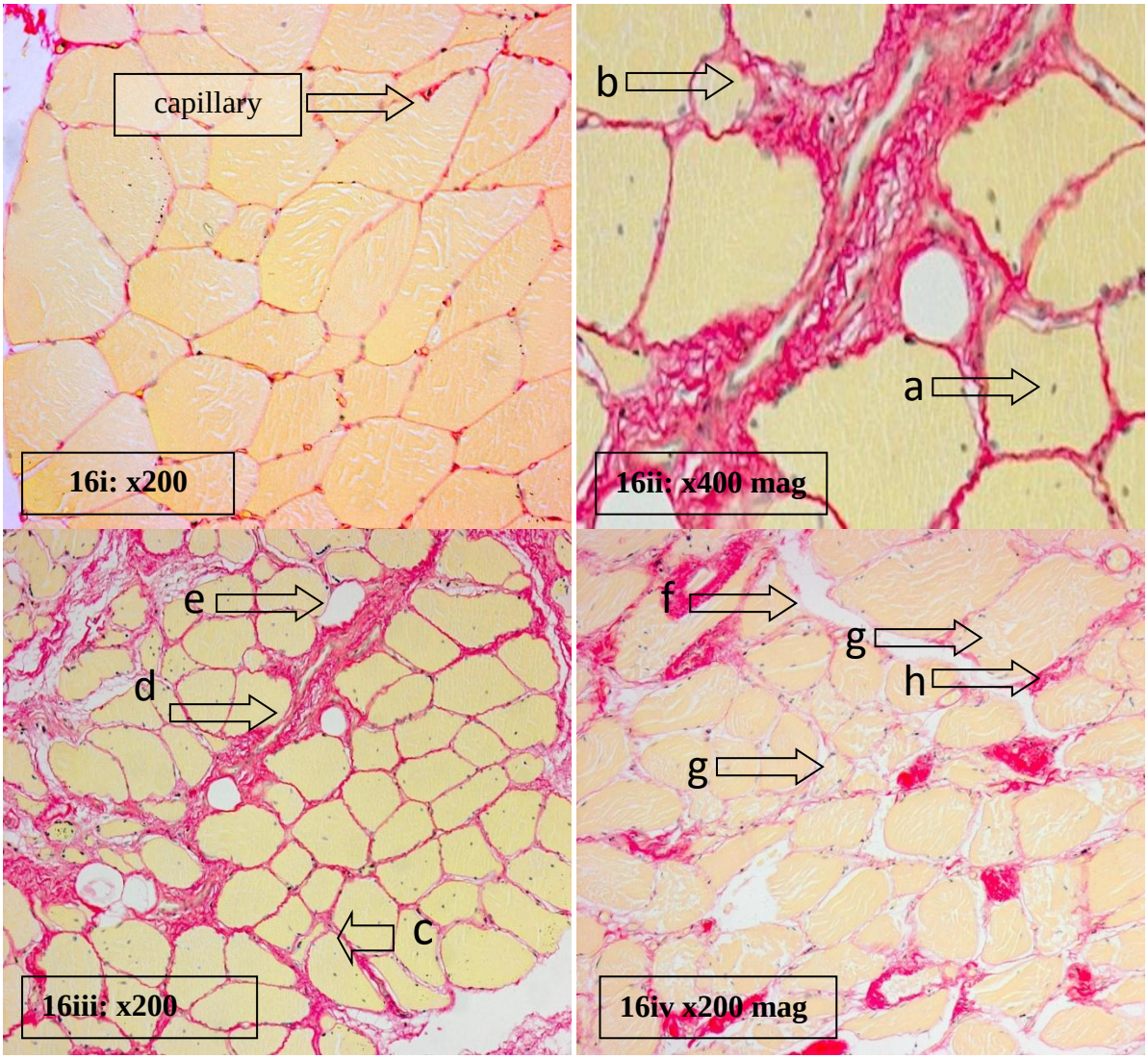


Figure 16: Picrosirius Red Staining of 16i: Control Muscle: uniform architecture with little collagen deposition, regular polygonal fibres, few central nuclei, rare small rounded fibres and flattened fibres. Capillaries can also be clearly seen surrounded by a thin collagenous compartment. Figures 16ii-iv: Picrosirius Staining of Ischaemic Muscle with: a. Central nuclei b. Small rounded fibres c. Flattened or angular Fibres d. Increased fibro fatty infiltration e. Increased adipose cell numbers f. Distracted and irregular tissue g. Increased Fibre Size Diversity h: Increased collagen around capillaries

4.2.3 Loss of Polygonal Structure is observed in Critical Ischaemia

70% (n=7) of ischaemic samples displayed these characteristics, yet only 10% (n=1) of the control group did so. This is in part perhaps due to the severity of the ischaemia affecting muscle innervation later in its natural history. Sample from patients with muscular dystrophy and an ischaemic sample have a similar loss of structure likely due to the effects of nerve damage common to both disorders (**see Figure 44**). There is the same atrophy of fibres, distraction of the normally tight syncytial arrangement, wide diversity of fibre size, as well as loss of polygonal structure.

The obvious fibro-collagenous overgrowth of the extracellular matrix is synonymous with ischaemic damage and also appears more commonly in severely ischaemic tissue samples than those of the control group who must have a degree of atherosclerosis on a microscopic level but have no clinical manifestations. Other features observed in the picrosirius red stained samples include the presence of adipose cells within the ischaemic samples as well as angular and small rounded cells.

As shown in Figure 17, there was a significant (p=0.0001; 95% CI) increase in the number of fibres seen per 400 x 300μm field across five different areas per sample in the ischaemic group compared to the control group indicating general atrophy and/ or increased regeneration of myofibres:

Table 9: Mean Number of Fibres/ 5 x Fields						
Control				**Ischaemia**		
	A	28.8			C	69.4
	B	32			D	84.2
	F	25.8			E	75
	I	27			G	37.8
	J	23.5			H	49.4
	L	24.6			K	18.2
	N	22.6			M	57.6
	O	16.2			R	32.4
	P	35.2			S	35
	T	21.6			U	45
Mean:		**25.73**		**Mean:**		**50.4**

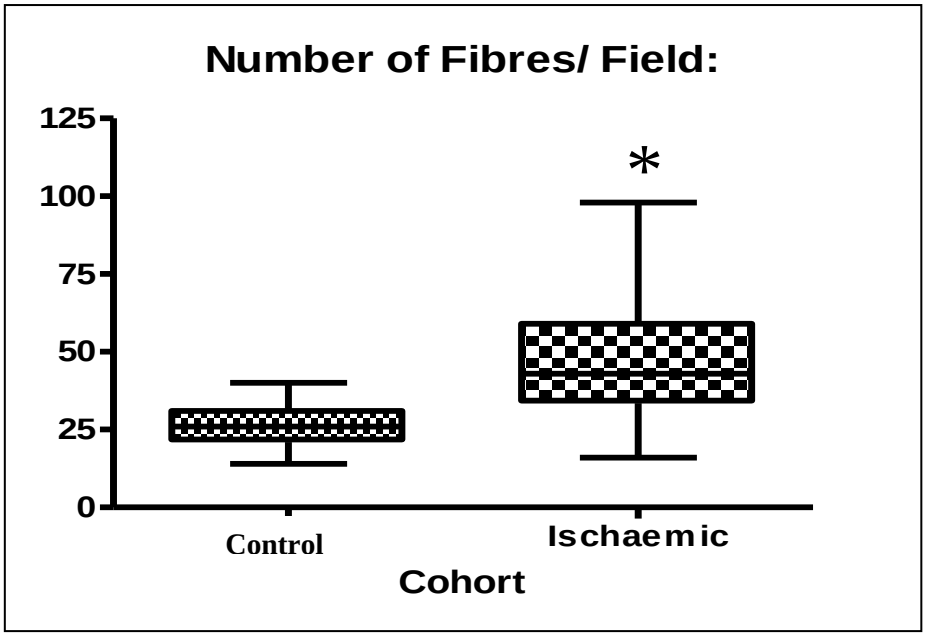


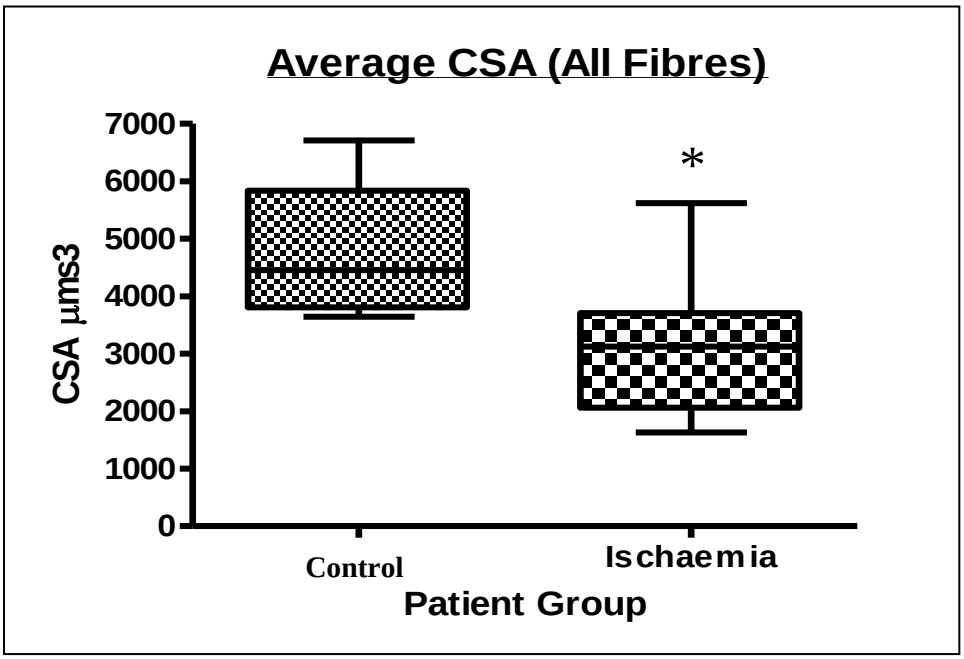
Figure 17: Table and Graph showing the mean number of fibres counted in five randomly determined fields per sample performed at x200 magnification. A significant difference was observed between the ischaemic vs control (CABG) group with more fibres seen per 400x300µms field; (*p=0.0001; 95% CI).

4.2.4 Cross Sectional Area of Myofibres is significantly reduced in CLI

Greater amounts of red staining collagenous tissue surrounding each myofibril, capillary and fascicle are seen in the ischaemic samples. Cross sectional area (CSA), as measured in 100 fibres per sample was found to be significantly greater in control samples than the more ischaemic samples (x1.5 fold; p=0.0147: 95% CI).

Table 10: Average CSA (All Fibres)						
Control				**Ischaemia**		
	A	3643.08			C	3901.1
	B	4215.51			D	2364
	F	4687.53			E	1628.64
	I	3929.25			G	3071.74
	J	6136.58			H	3418.47
	L	3782.83			K	5621.05
	N	5532.44			M	1661.21
	O	6709.32			R	3171.94
	P	3732.51			S	3508.46
	T	5201.86			U	2719.81
Mean:		**4757.091**		**Mean:**		**3106.642**

Figure 18: Table and Graph to illustrate the mean cross sectional area (CSA) of 100 fibres per patient biopsy measured at x400 magnification. x1.5 fold significant decrease (* p=0.0147; 95% CI) in the CSA of ischaemic myofibrils was observed compared to the Control Group showing not only general atrophy of fibres but also increased regeneration of fibres occurring.



Greater diversity of size of the fibres in the ischaemic muscle was observed, as well as evidence of ongoing atrophy, regeneration and denervation. An increase in fibrofatty infiltration can be observed with loss of the normal polygonal architecture of the muscle fascicles.

4.2.5 Central Nuclei Are Indicative of Regenerating Muscle

Central nuclei, a feature of regenerating myofibres, were observed in both ischaemic and control samples with no significant difference in frequency observed between the two cohorts.

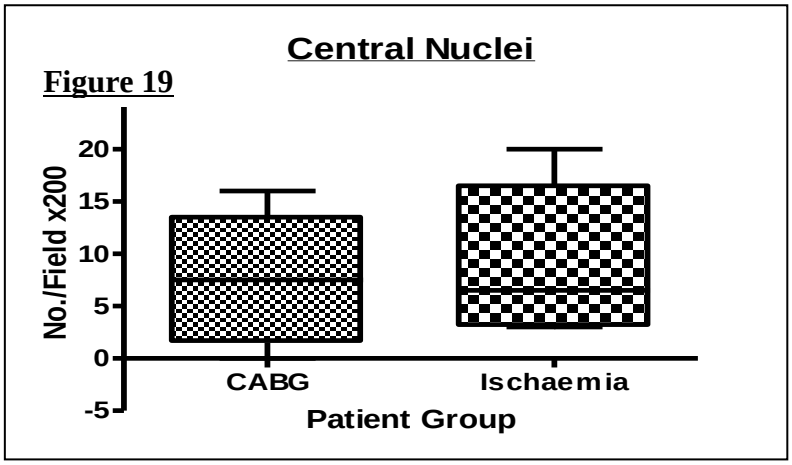


Figure 19: Graph to illustrate the mean number of central nuclei observed in 100 myofibrils per biopsy sample counted at x200 magnification. There was no significant difference between the two groups

4.2.6 Myonuclei Number Remains Static Despite Fibre Loss and Atrophy

Certainly at first glance there is an abundance of nuclear material evident in ischaemic tissue. However, most of these nuclei belong to migratory cells, in particular leucocytes, macrophages and neutrophils. Importantly, although there was established atrophy of ischaemic fibres as detailed earlier in this section, the number of myonuclei per fibre identified at x400 magnification for 100 fibres per sample, did not differ significantly despite the previous observations in atrophied muscle (100;100;203), although this loss has been reported in soleus. However, the static number of myonuclei has been a reported phenomenon seen in hypertrophy of muscle (150).

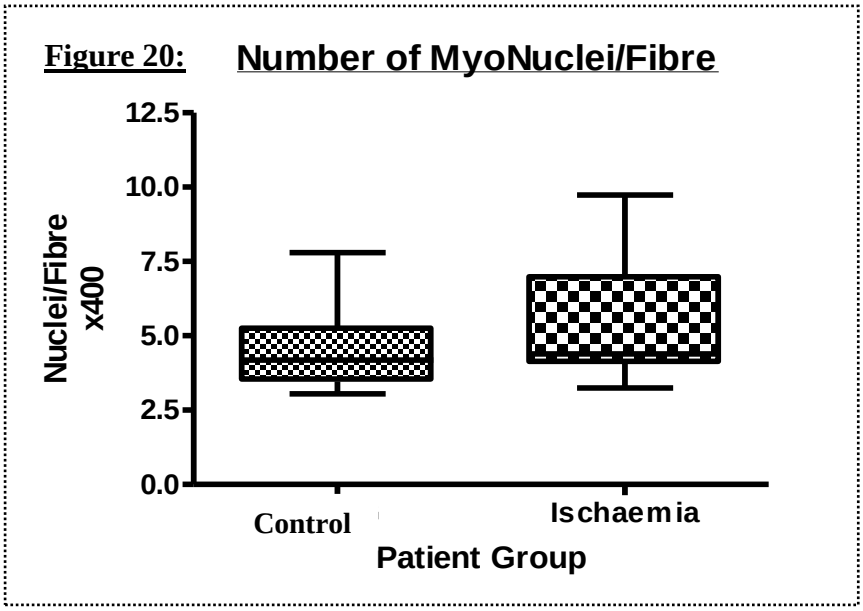


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Figure 20: Graph to show the mean number of myonuclei counted per 100 fibres per patient biopsy measured at x400 magnification in both cohorts. Interestingly, there was no significant difference between the two groups.

4.2.7 Angular fibres are indicative of denervation and are observed in greater numbers in CLI

Angular fibres, as seen in Figure 15, however, were significantly raised in ischaemic muscle with a tenfold increase (*p=0.0002: 95% CI).

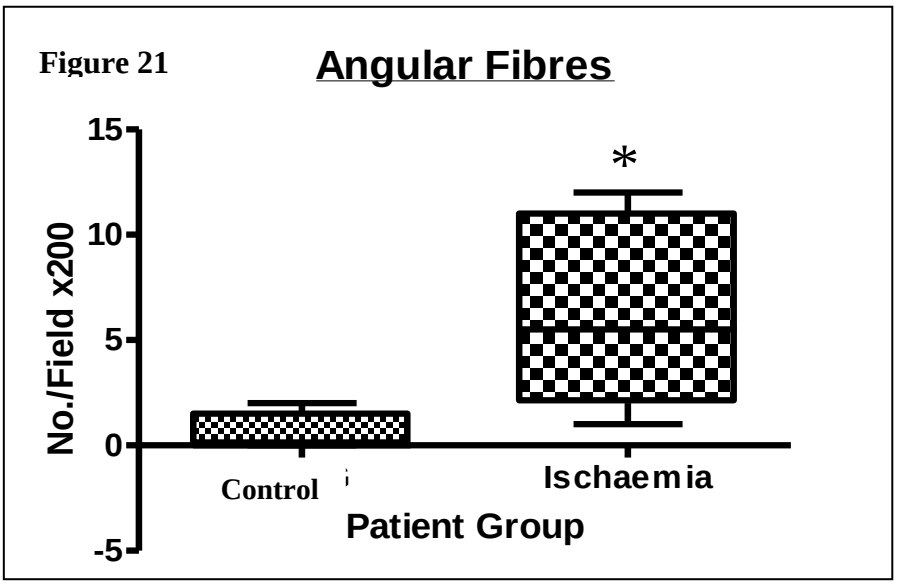
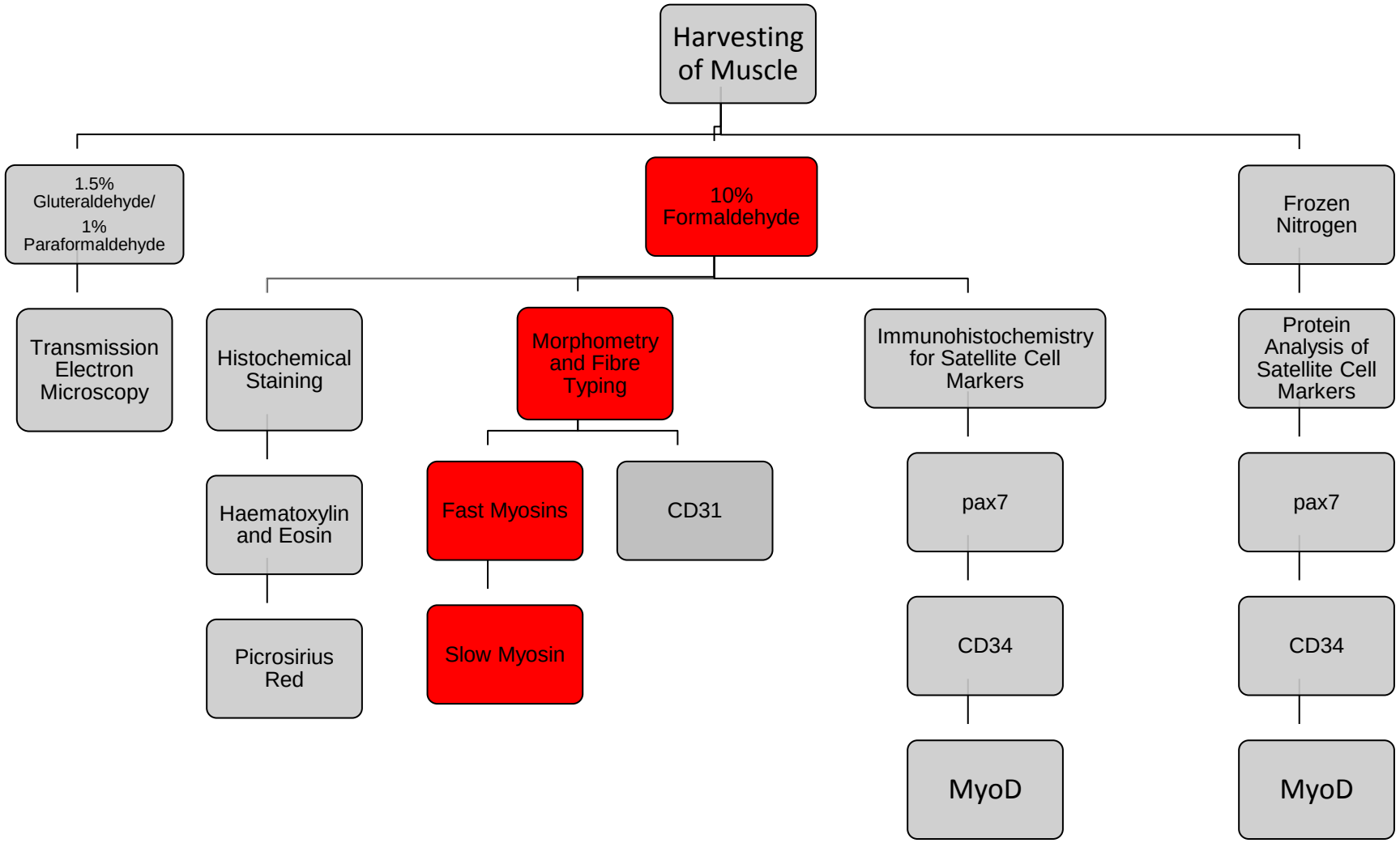


Figure 21: Graph to show the mean number of angular fibres occurring within 100 fibres assessed per patient biopsy, collected at x200 magnification. A x2 fold significant increase in the occurrence of angular fibres was observed in the CLI patient samples compared to the Control Group; (*p=0.0002; 95% CI).

4.3 **Figure 22:** Fibre Typing using Sequential Slides



Slides were stained in sequence (as described in **Section 3.6**) and shown in **Figure 23** below. Sequential slides reacting with DAB chromagen were typed as described previously (**Section 3.5**).

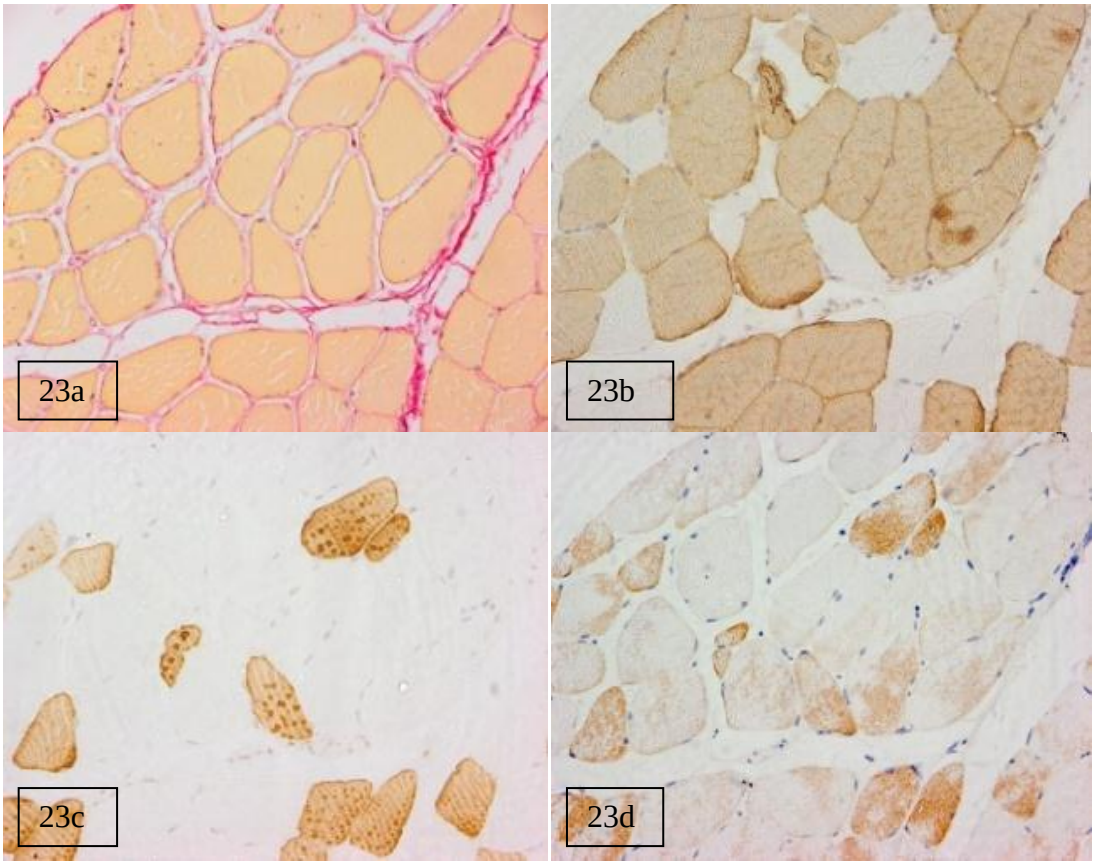


Figure 23: Sequence of Staining for Morphometry (after initial H&E stain for orientation). 23a: Picrosirius Red Showing Black Nuclei, Yellow cytoplasm and Red collagen. 23b: Slow Myosin (M8421) staining all Type I and Type IIc fibres brown.23c: Fast Myosin (A4.74) staining Type IIa MHC dark brown and Type IIx MHC light brown. 20d: Fast Myosin (sc71) staining all Type II MHCs brown.

4.3.1 Reliability of Fast Myosin Antibodies (sc71 and A4.74)

Recent studies have suggested that sc71 is specific for IIa only yet A4.74 also weakly stains type IIx fibres when subjected to microwave antigen retrieval with pH=9 TRIS-EDTA. This reported staining was however observed in fixed frozen tissue and has yet to be reported in paraffin embedded sections (57;177;185).

There was no significant difference in staining between the two fast antibodies in recognising type II fibres overall. The reported differentiation in intensity of staining of type IIa and type IIx in frozen muscle by sc71was not reproducible in this study, which used formalin fixed paraffin embedded tissue.

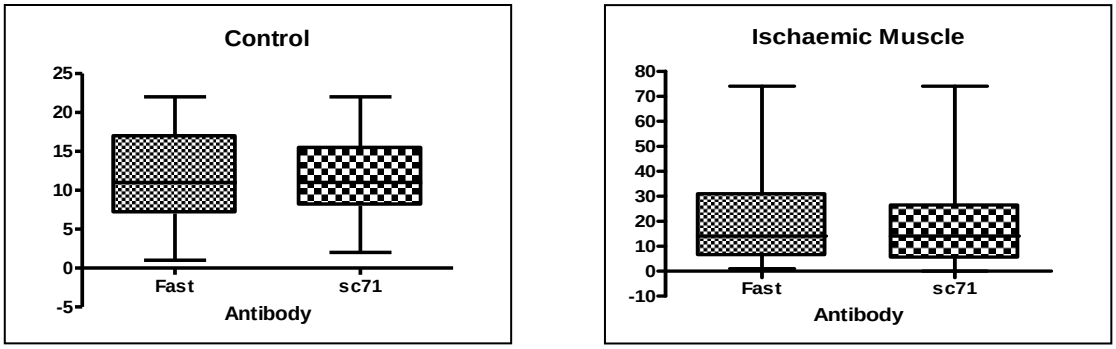


Figure 24a: A4.74 (Fast) specificity compared to sc71 anti-fast MHC in both Control (CABG) and CLI tissue

The A4.74 fast anti-myosin antibody however was able to reliably distinguish between the pure fast myosin fibres with IIa staining more strongly and IIx less so.

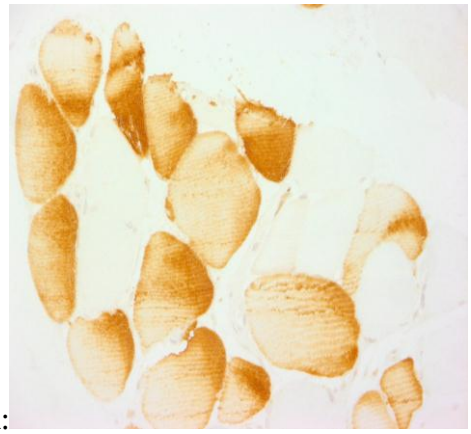
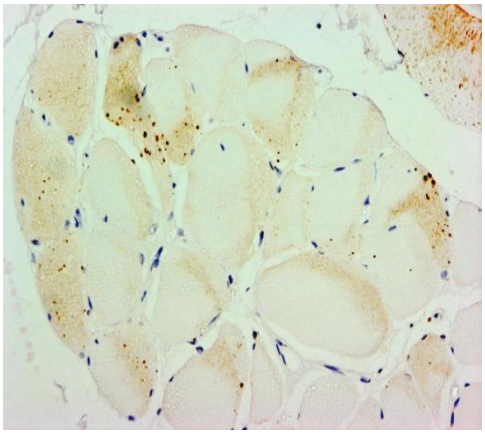
25a:  **25b**: 

Figure 25: Intensity of Fast Myosin Stains: a:A4.74 stain b: sc71 stain; A comparison of the pattern of myosin staining showed similar specificity in labeling fibres, but background and poor signal in sc71 were such that it was unreliable to gauge a weak or strong chromagen expression for the purposes of identifying IIa or IIx MHC.

4.3.2 Fibre type proportion display a shift towards type I in CLI

The proportions of each fibre type; I, IIc, IIx and IIa but except IIa/IIx, were recorded in a total of 100 fibres per patient sample in three different fields and the two cohorts were compared as described previously. The graphs below show the relative proportions of myosin heavy chains.

In this study, there was a significant shift (p=0.0288; 95% CI) in fibre proportion to slow myosin, or type I fibres by 75%. Most interestingly, there was a significant increase in the number of hybrid I/IIa or IIc fibres in the ischaemic samples. On average there was a 2.4 fold rise in these fibres (p=0.0147; 95% CI). These relative increases in type I fibres and hybrid IIc fibres seem to be at the expense of IIx fibres, which were significantly less common in ischaemic tissue. Six times fewer type IIx fibres were seen in ischaemic tissue (p=0.0039; 95% CI). There was no significant difference in the proportion of IIa fibres or those fibres that did not stain and were therefore unidentifiable.

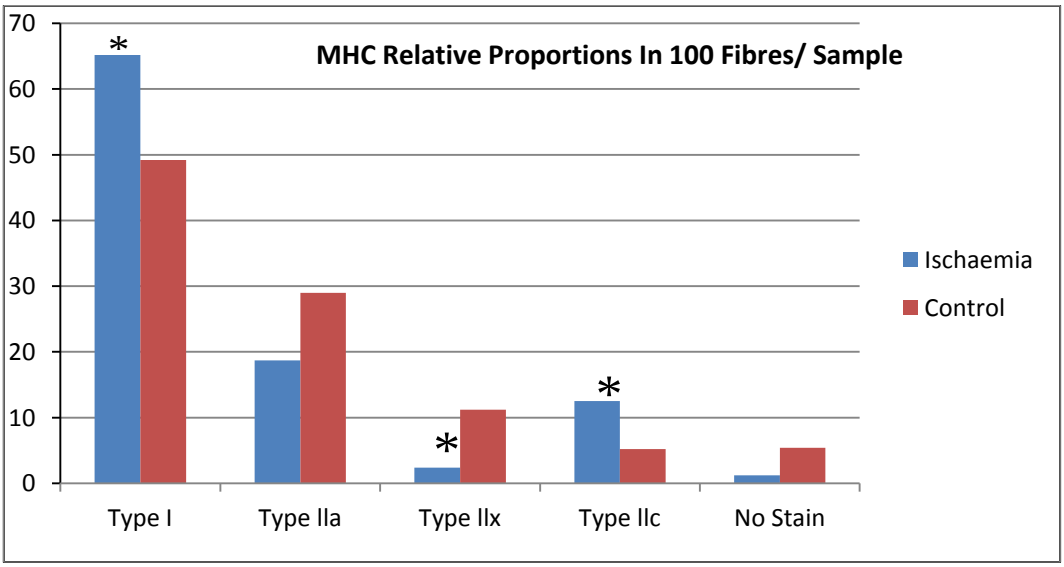


Figure 26: Mann Whitney U test to assess relative proportions of MHC expression in 100 Fibres per sample in ischaemia vs control samples: There was a significant shift (p=0.0288; 95% CI) to type I fibres by 75%. There was 2.4 relative rise in IIc fibres (p=0.0147; 95% CI). IIx fibres were six times less abundant in ischaemic tissue (p=0.0039; 95% CI). There was no significant difference in the proportion of IIa fibres or those fibres that did not stain.

% Fibre Type Proportion
Control vs CLI

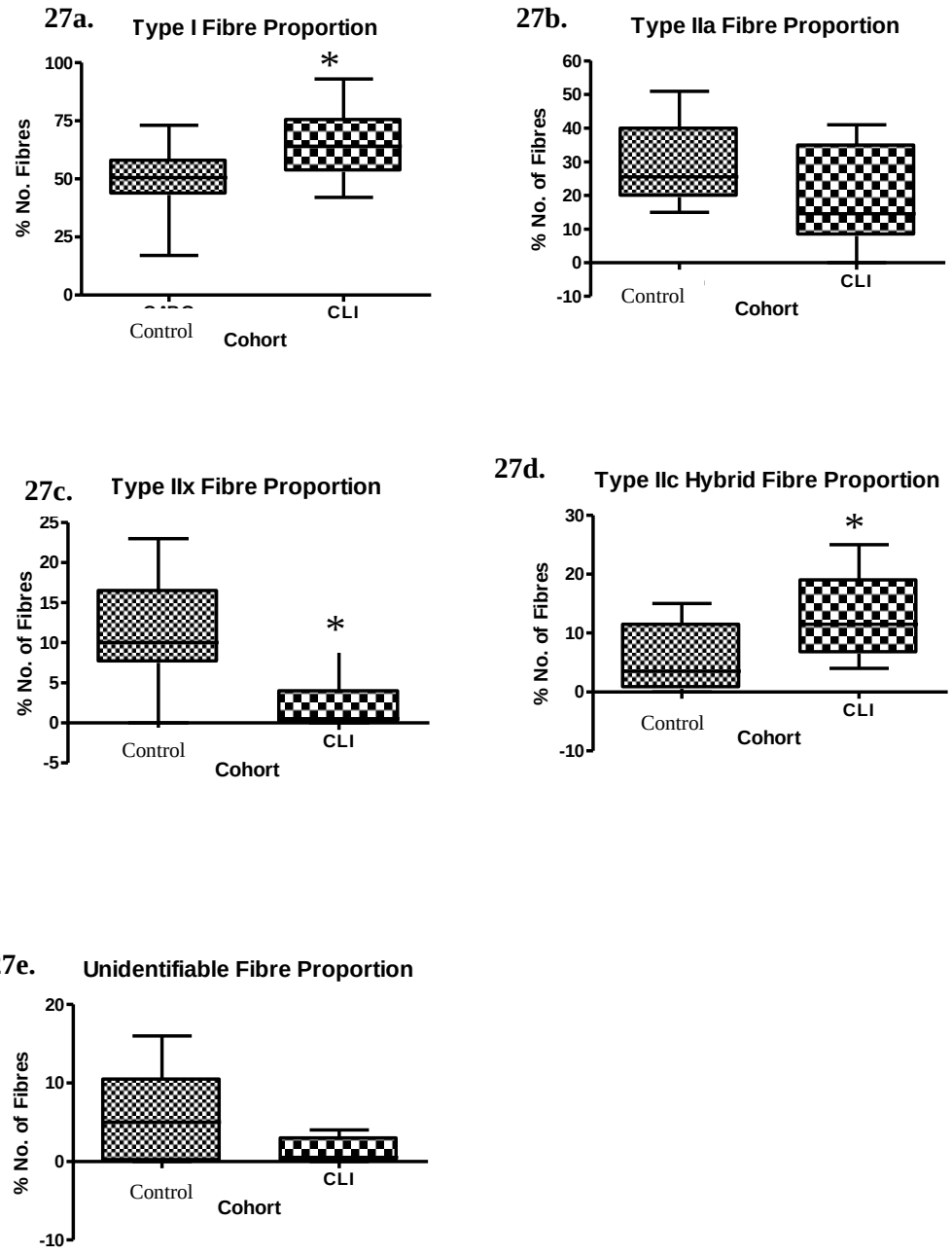


Figure 27: Showing the separate relative fibre type proportions (%) present in Control (CABG) patients Vs CLI patients. 27a. Type I slow MHC fibre proportion was significantly raised in ischaemia (*p=0.0288; 95% CI). 27b. Type IIa Fast MHC fibres were unaltered significantly. 27c. Type IIx staining fibres were significantly decreased in ischaemia (p=0.0039; 95% CI). 27d. Type IIc fibres were significantly increased in ischaemia (*p=0.0147; 95% CI). 27e. Unidentifiable Fibres were unaltered in proportion in ischaemic muscle

4.3.3 Individual fibre type atrophy in response to CLI

Muscle sections were harvested and immediately preserved in formalin and wax embedded, not only because this technique affords better preservation of the architecture but also for the following reasons laid out by Behan: "[Formalin fixed tissue] has other advantages [that] include the ability to study both fibre types on the same preparation, economy of use, and the production of permanent and colourful preparations so that image analysis is easy. Economy of tissue use is important now that needle biopsies are becoming routine and micromethods for all parameters, including gene analysis, are available (193)."

After identifying 100 random fibres per sample and classifying them according to their type, each cross sectional area was measured at x400 magnification allowing fibre cross sectional area (CSA) to be assessed for each fibre type in both ischaemic and control groups and later analysed.

Of the fibres that did not stain for all antibodies and were therefore unidentifiable, there was no significant fibre atrophy with no statistical difference between the size of the fibres in control nor ischaemic muscle ($p=0.0892$)

Type IIc fibres were found to be the only other fibre group that showed no significant change in fibre diameter in either group ($p=0.315$) and therefore are the least susceptible to fibre atrophy. All other identifiable groups, i.e. types I ($p=0.0185$; CI=95%), IIa ($p=0.0089$; CI=95%) and IIx ($p=0.0029$; CI=95%) all showed a significant decrease in fibre size. However, of these, type I fibres were the most resistant to atrophy. Type IIx as previous

studies have suggested was the most susceptible to damage due to ischaemia and indeed displayed the greatest atrophy across all fibre types.

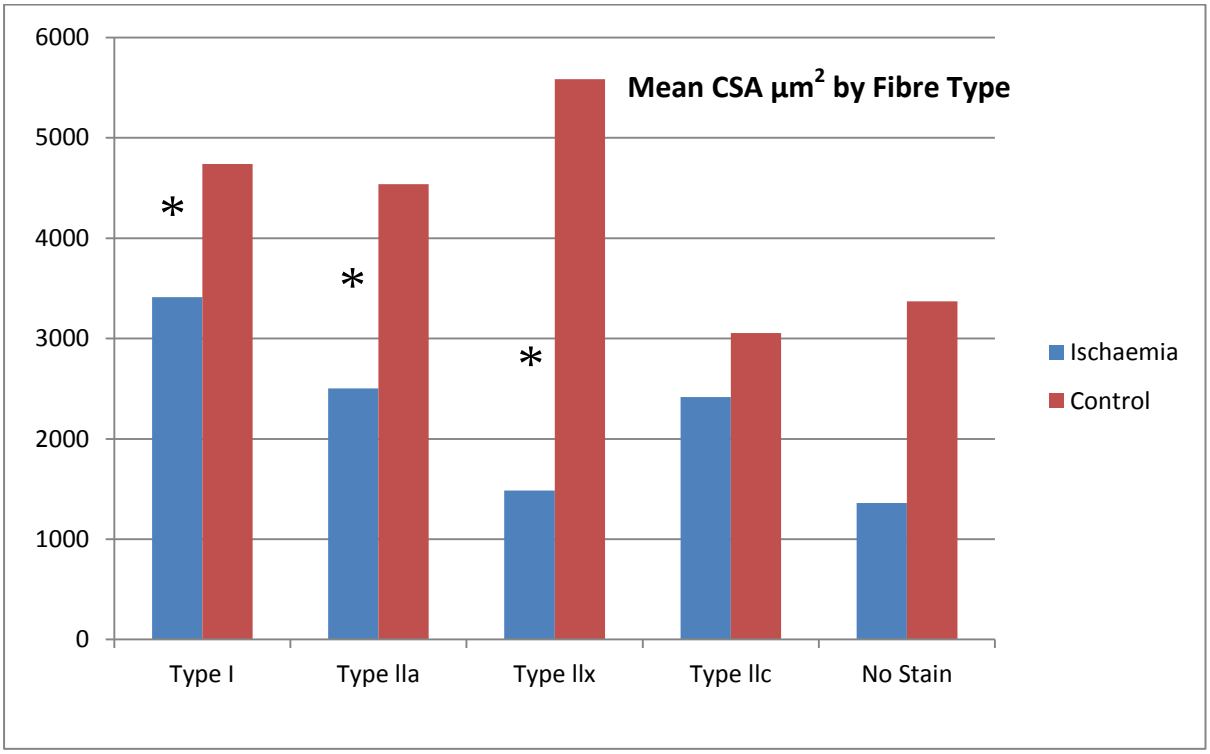


Figure 28: Significant Atrophy was demonstrated in all fibre types except for type IIc hybrid fibres. Type I (p=0.0185; CI=95%), IIa (p=0.0089 CI=95%) and IIx (p=0.0029; CI=95%) all showed a significant decrease in fibre size, Type IIc and unidentifiable fibres did not show a significant change in CSA and may therefore be more resistant to ischaemia induced atrophy. This data was analysed using the Mann Whitney U Test.

Cross Sectional Area By Fibre Type

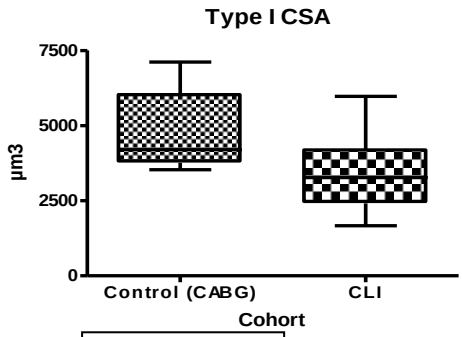
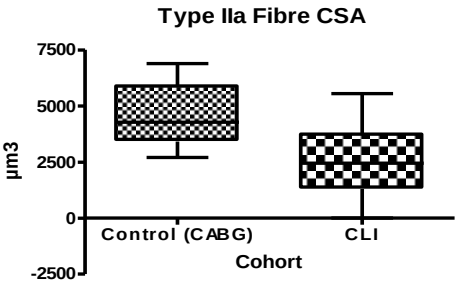


Figure 29a:



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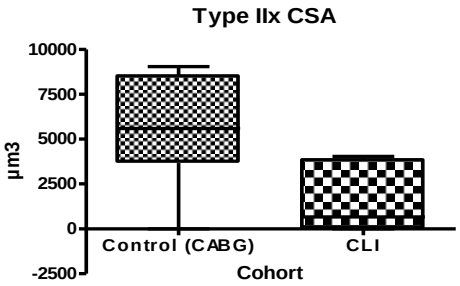

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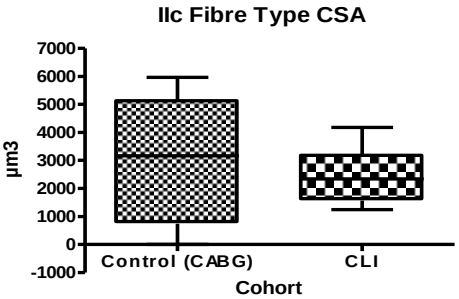


Figure 29d:

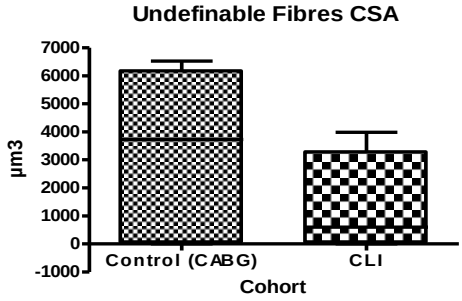
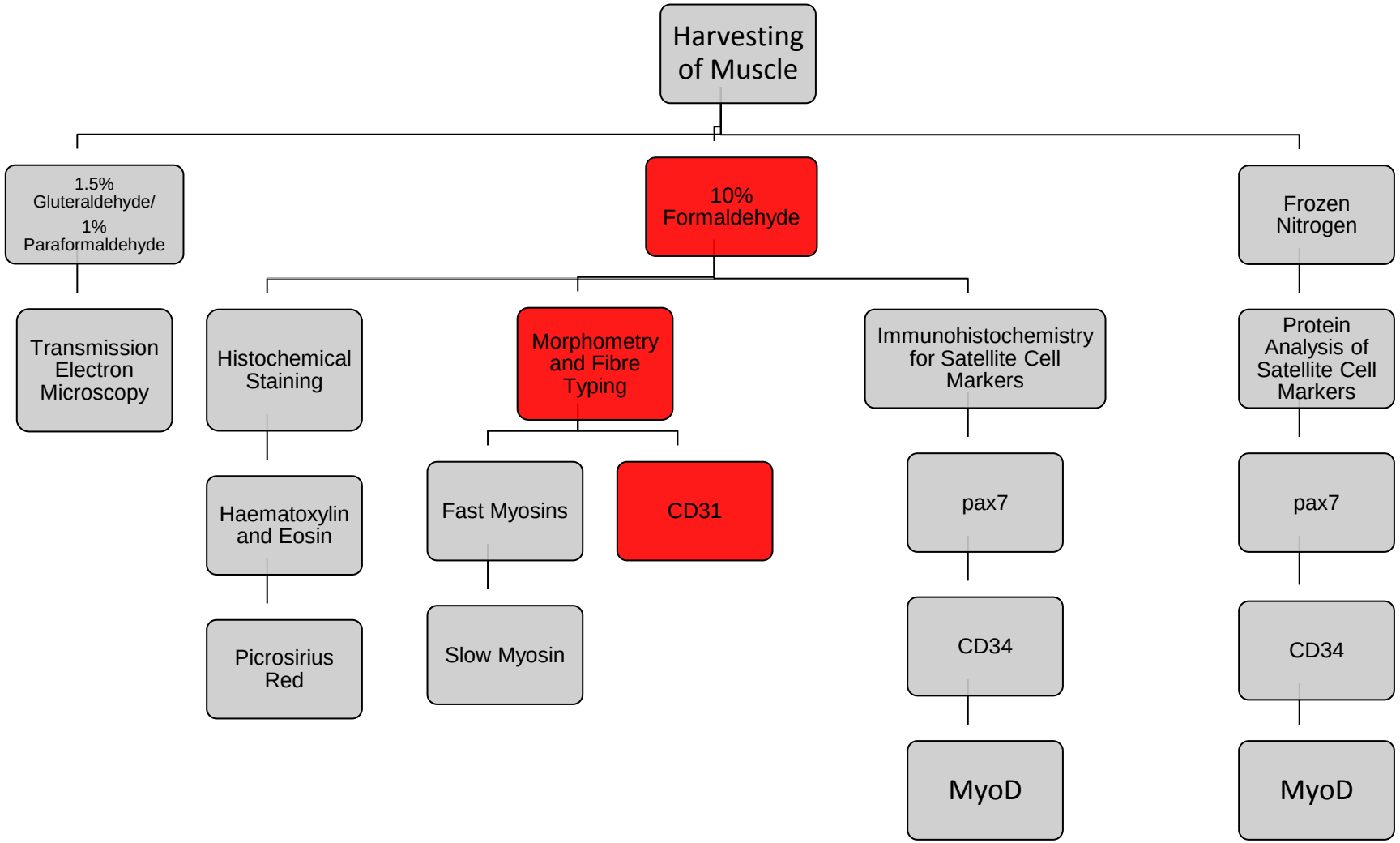


Figure 29e:

Figure 29: Mean CSA (μm^2); Comparison between Control and Ischaemic Tissue. 29a:Type I fibres significantly reduced in CSA (*p=0.0185; CI=95%), 29b:Type IIa Fibres significantly reduced in CSA(* p=0.0089 CI=95%). 29c: Type IIx Fibres significantly reduced in CSA (*p=0.0029; CI=95%) 29d: Type IIc Fibres did not display a significant change in size in ischaemia, 23e: Unidentifiable fibres did not significantly alter in size in ischaemia.

Figure 30: Angiogenic Response in CLI



4.3.4 Endothelial Cell Density Is Increased In CLI, Indicating a Stimulated Angiogenic Response

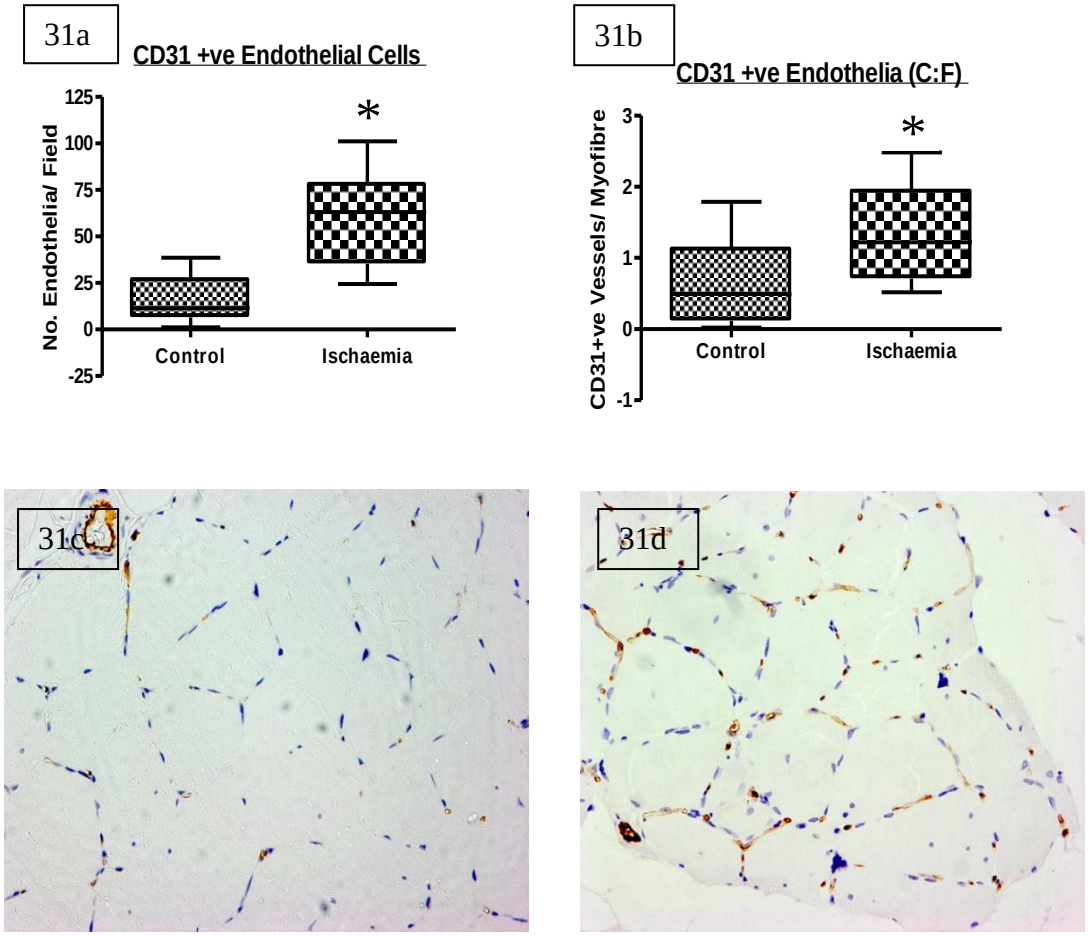


Figure 31: 31a. CD31 Microvessel Density in CLI: significantly increased in ischaemic muscle (*p=0.0001). Figure 31b: Microvessel/ Myofibre Density significantly increased in ischaemic tissue (*p=0.035). Figure 31c: CD31 expression of endothelial cells in control muscle compared to 31d: CD31 expression in ischaemia muscle, the latter displaying many more positive cells.

CD31 is a marker of continuous endothelia and establishing capillarisation in muscle is an important part of morphometric analysis. Positive staining cells were counted in five fields, measuring 400 x 300μms, for each sample at x200 magnification, these fields being those analysed sequentially after the previously fibre typed fields. A clean and reliable stain was

achieved with an obvious increase in the number of CD31 positive cells. This was quantified as a 3.7 fold increase in the density of CD31 positive nuclei ($p=0.0001$) in the ischaemic samples compared to the control group.

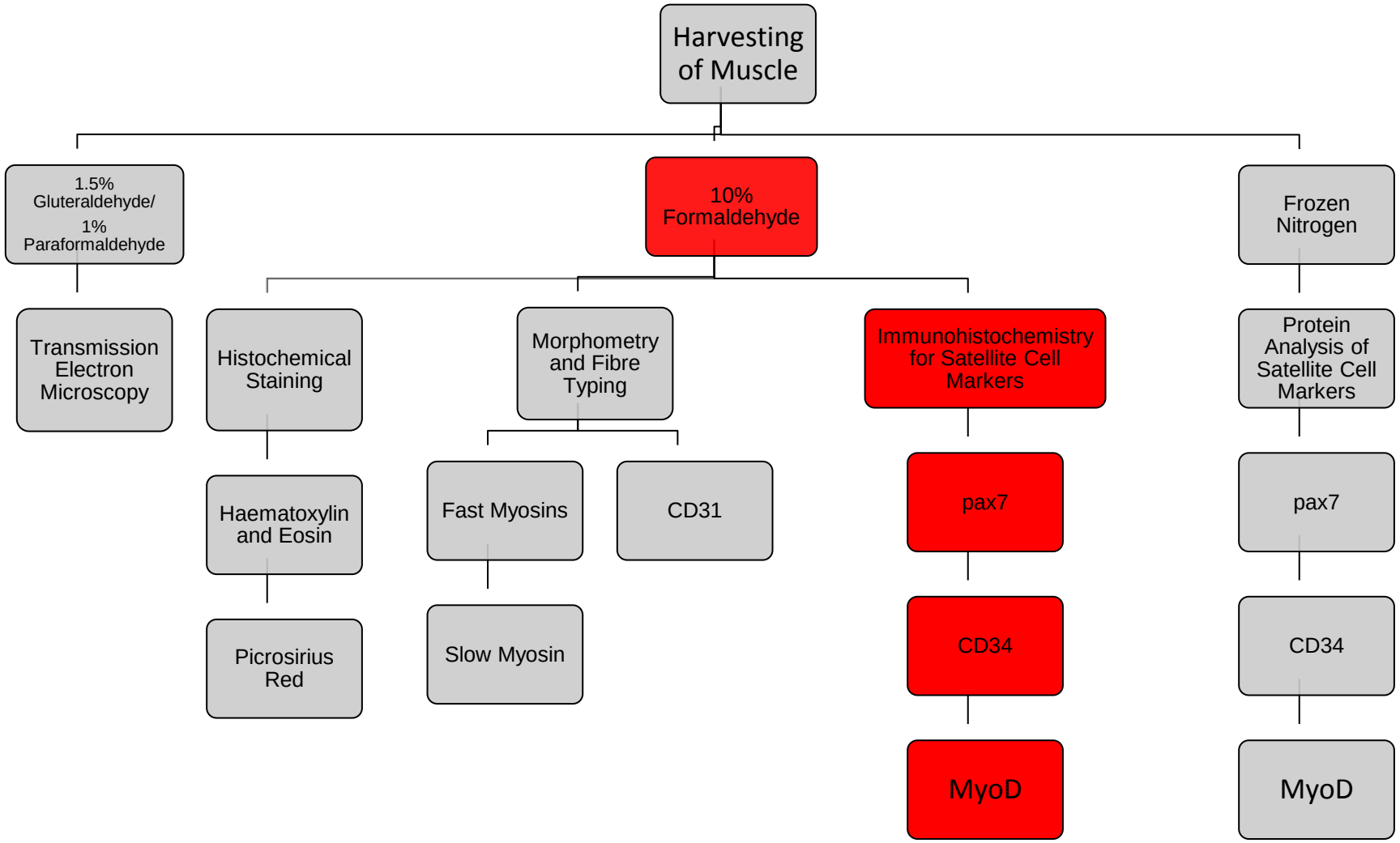
To ensure that there were not more positive cells per field simply due to shrinkage of tissue due to the general atrophy of the muscle fibres and the extracellular matrix, the number of cells per myofibre was also calculated. Despite the significant increase in number of fibres per field discussed earlier, there was still a significant increase in the number of CD31 positive cells by over two fold ($p=0.035$).

The parameters in general use by researchers to assess fibre morphometry and thus fibre type changes include; overall calf muscle circumference, total fibre number, type specific fibre numbers (often expressed as a % of the total), fibre type area (% coverage of each MHC isoforms/ total fibre area), individual fibre size and fibre size diversity, connective tissue cover, capillary to fibre ratio (C:F), the number of capillaries in contact with each muscle fibre, location of central nuclei (regenerating fibres), angular/ flattened fibres, small rounded fibres (regenerating) and fibre type grouping known as clumping, indicating reinnervation.

4.3.5 Summary of Morphometry Findings Observed In CLI

- Loss of tight polygonal architecture.
- More fibres/ field.
- Increased adipofibrocytes.
- Increased collagen.
- Increased capillarisation.
- Increased cell size variability.
- Increased incidence of fibre clumping.
- Angular cells indicating denervation are more frequent.
- Central nuclei, indicative of regenerating myoblasts, are no more abundant in CLI
- Myonuclei number per fibre is not affected by CLI
- Type I and IIc MHC fibre proportions significantly increase.
- Type IIx proportion significantly decreases.
- Type IIa fibre proportion stays the same.
- Significant atrophy demonstrated by decreased CSA in I, IIa and IIx fibres
- Type IIc hybrid fibres do not display significant atrophy.
- Endothelial cell presence in ischaemia is significantly increased.
- Some fibres remain unidentified.
- Type IIa/x hybrid fibres remain unaccounted for.
- BF35, neonatal and embryonic myosins were undetected.
- Electron microscopy can be used to differentiate between active and quiescent satellite cells.

4.4 **Figure 32**: Sequence staining of satellite cell markers



4.4.1 Satellite cell numbers as determined by pax7 staining are significantly increased in CLI muscle

The majority of satellite cells express pax7 and previous studies show pax7 is not expressed by any other cell type in adult human skeletal muscle. This makes using pax7 alone as a sole marker of satellite cells tempting. There was a certain number of positive nuclei expressing pax7 in control tissues (mean = 21.8 nuclei staining for pax7/field) and these were certainly specific with little background staining.

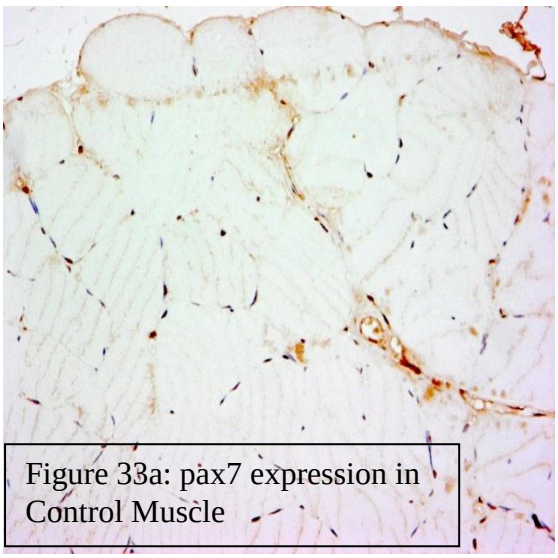


Figure 33a: pax7 expression in Control Muscle

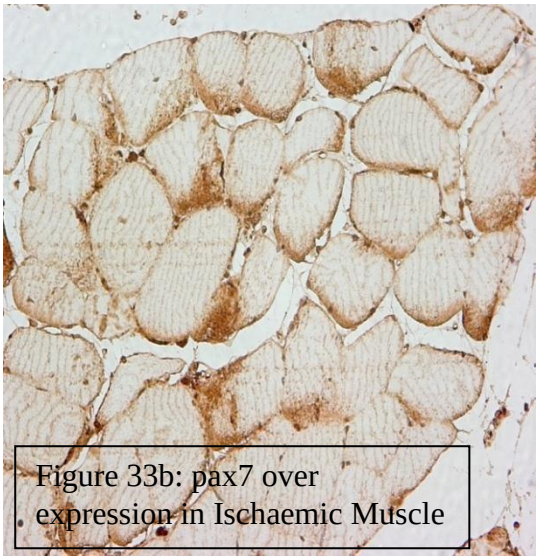


Figure 33b: pax7 over expression in Ischaemic Muscle

Figure 33: Anti-pax7 antibody positive staining indicating total satellite cell numbers in 33a: Control vs 33b: Ischaemic muscle. In CLI samples, there was marked over expression of chromagen throughout the muscle tissue. This was not apparent in the Control samples analysed.

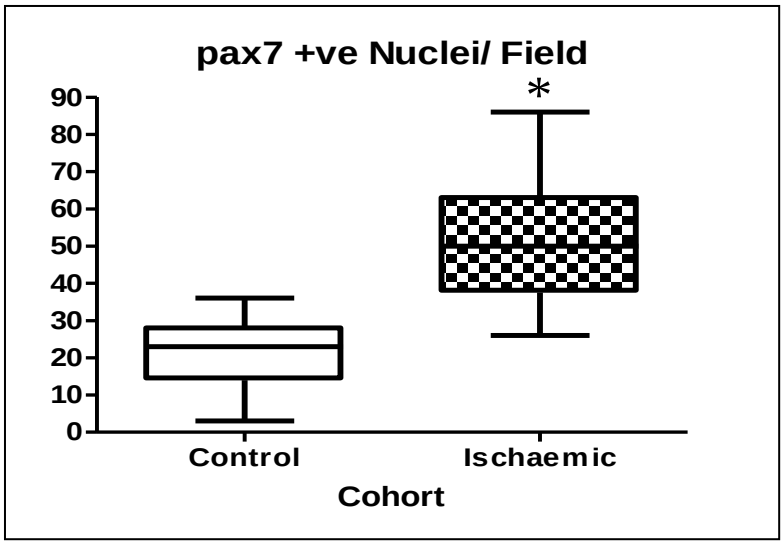
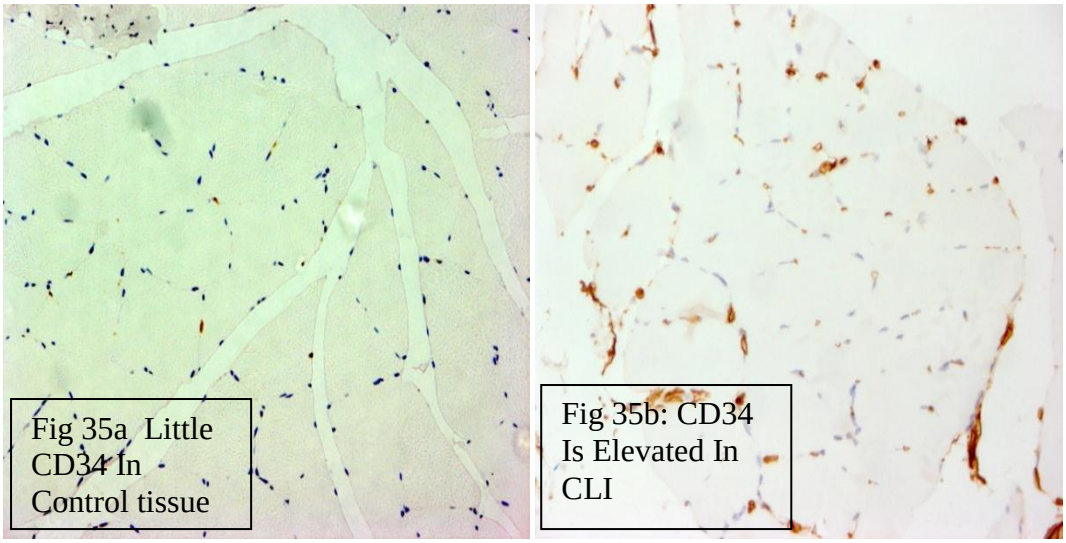


Figure 34: Graph Showing Mean Frequency of Pax7 positive Nuclei in Ischaemic vs Control Muscle samples: per 5 random sequential 400x300μms fields/ patient biopsy There was a significant x2.4 fold increase p<0.0001; 95%CI.

There was a significantly higher expression of pax7 throughout the ischaemic samples with apparent over expression. Those nuclei clearly staining positive for the transcription factor were also found to be significantly raised, with a x2.4 fold increase seen in ischaemic tissue (p<0.0001; 95%CI). A typical sample of each is shown in figure 33 above:

4.4.2 Haematopoietic Stem Cells (HSCs) are present in greater numbers in CLI tissue:

Stem cells capable of forming blood cells are defined as haematopoietic stem cells or HSCs (204) and this group of cells was identified using the CD34 antibody (186;205;206) and are the precursors of many types of mesodermal cells in adult skeletal muscle including neural, muscle and adipofibrocytes. Their daughter cells, myogenic precursor cells or satellite cells are capable of self renewing the satellite cell pool or forming myoblasts, although other research groups have postulated that it is a separate subset of satellite cells that are in fact responsible for renewing the satellite cell pool, these are all still identifiable using an anti-CD34 stain (186). It has also been suggested that this renewal of the satellite cell population is due to circulating haematopoietic cells derived from bone marrow or blood (158) and indeed these cells have been proven to be multipotent and capable of forming many different mesodermal progeny, including blood cells (red, white, platelets), endothelial cells, skeletal muscle cells and immune system lineages.(186;204;207;208)



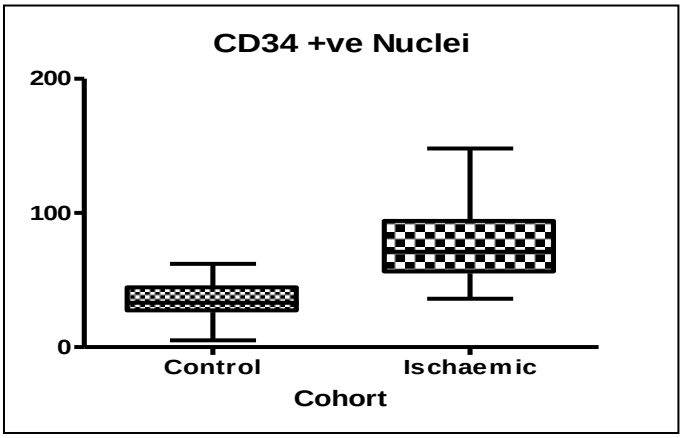


Figure 35: CD34 positive staining nuclei in 35a. Control Tissue compared to 35b. Ischaemic Tissue after immunostaining; mean number per five random sequential fields per sample. Figure 35c Graph to demonstrate significantly more CD34+ve nuclei in Ischaemic tissue (*$p<0.0001$) compared to Control tissue indicating a twofold rise in HSCs and quiescent satellite cells. CD34 staining was specific and the number of positively staining nuclei per five 400x300μms fields at x200 magnification were assessed per patient biopsy. There was a two fold increase ($p<0.0001$) in the number of CD34 positive cells observed staining in the ischaemic tissue compared to the control tissues.

4.4.3 Expression of MyoD in ischaemic adult human skeletal muscle is significantly reduced

MyoD, a marker of myoblast and activated satellite cells, but not myotubes nor mature muscle cells (209;210) as used to assess myogenic activity in muscle samples. There was a significant decrease x1.75 in those satellite cells staining for myoD in ischaemic tissue ($p<0.0001$). This shows that while pax7 is evident in promoting cell proliferation, fibre type shift and haemopoietic stem cell recruitment, pax7 does not lead to an increase in myoD in satellite cells and there is in fact reduced differentiation. In fact, there appears to be an inhibitory effect on myoD by pax7 (148).

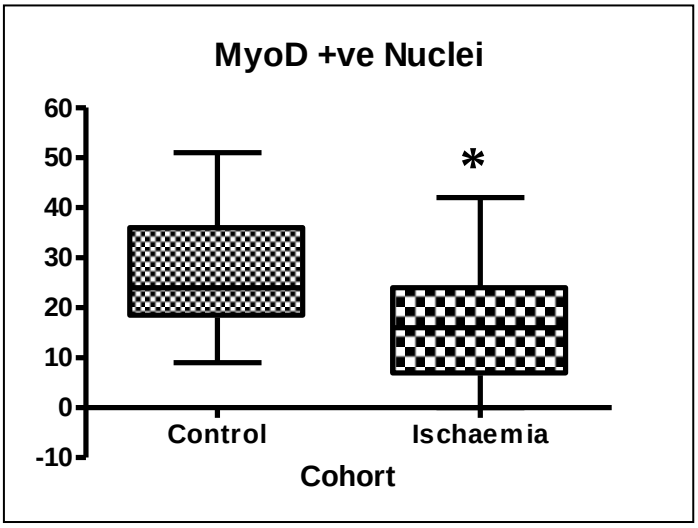


Figure 36: Graph representing mean number of MyoD positive satellite cells per 5 random sequential 400x300μms fields/ patient biopsy in control vs ischaemic tissue. There was a significant decrease in MyoD expression in the ischaemic muscle when compared to control tissue = x1.75 $p<0.0001$)

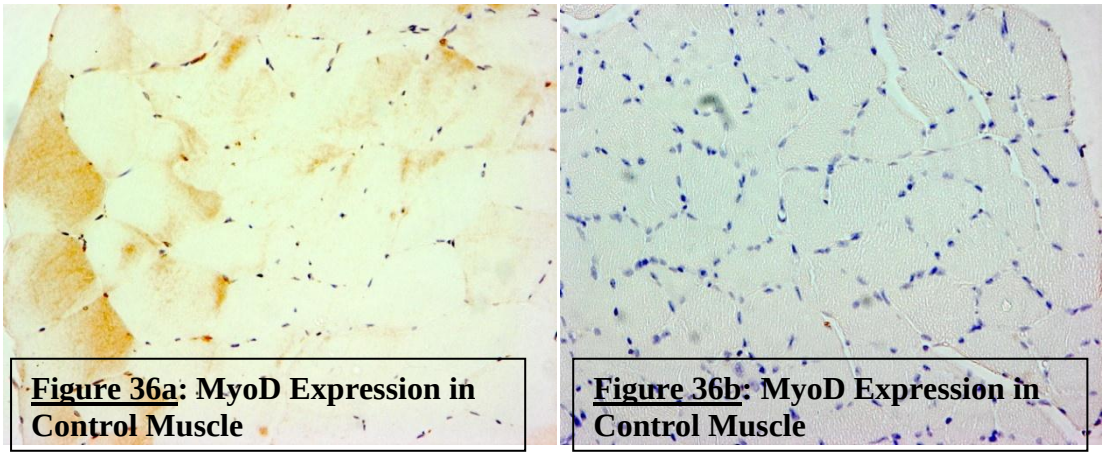


Figure 36a: MyoD Expression in Control Muscle

Figure 36b: MyoD Expression in Control Muscle

Figure 36: MyoD Expression in 36a: Control vs 36b: Ischaemic Muscle Tissue (x200 Magnification) MyoD cytoplasmic staining indicates differentiating myoblasts and nuclear staining highlights active satellite cells. There was almost no chromagen expression in the ischaemic tissue (36b).

4.4.4 Pax7 and CD34 co localize to show a significant increase in quiescent satellite cell numbers on sequential slides in CLI tissue

As co-localisation of CD34 and pax7 via double staining was unsuccessful, the fact that both these antibodies had been developed with DAB on sequential slides still allowed for the recognition of quiescent satellite cells by using x200 magnification photographs of the same five 400x300μms fields previously stained separately with pax7 and CD34. These were superimposed upon each other and the number of cells, quiescent satellite cells, staining positive for both pax7 and CD34 were compared across the two patient groups.

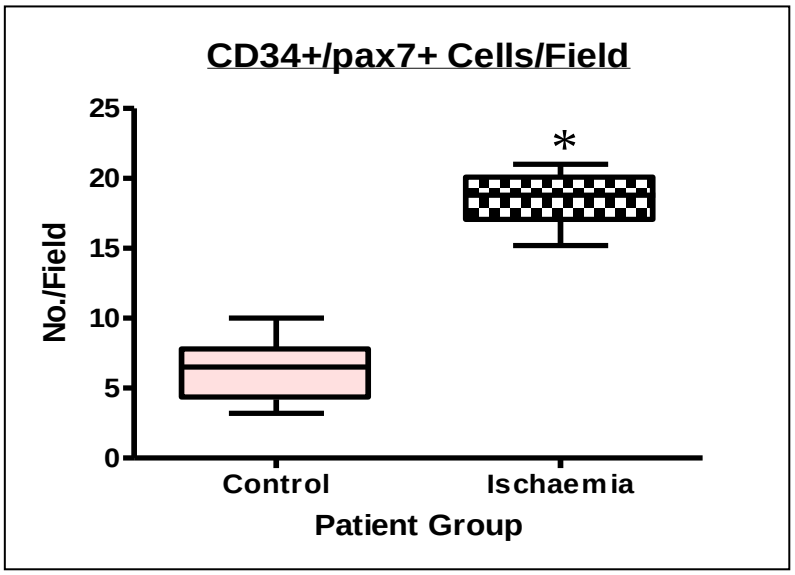


Figure 37: Mean number of quiescent or pax7 +/CD34 +/ MyoD - satellite cells_as per 5 400x300μms fields of control vs ischaemic muscle; There were significantly more satellite cells, a 2.9 fold increase in ischaemic muscle, staining positively for CD34 and pax7 *p<0.0001: 95% CI

There was a mean of 6.3 cells staining positive for both CD34 and pax7 per 400x300μms field in control tissue compared with 18.46 cells per field in ischaemic tissue, a x2.9 fold increase (p<0.0001).

4.4.5 Pax7 and MyoD co expressing nuclei are indicative of active SCs and are significantly reduced in ischaemic compared to control muscle

MyoD and pax7 are co-expressed by active satellite cells (159;211;212). Using the same methodology that was applied to counting the pax7/CD34 positive cells, JPG photographs previously taken of sequential sections stained for pax7 and myoD were superimposed upon each other. The number of cells staining in five fields was recorded as a mean number for each patient and was analysed using the Mann Whitney U test at 95% confidence intervals.

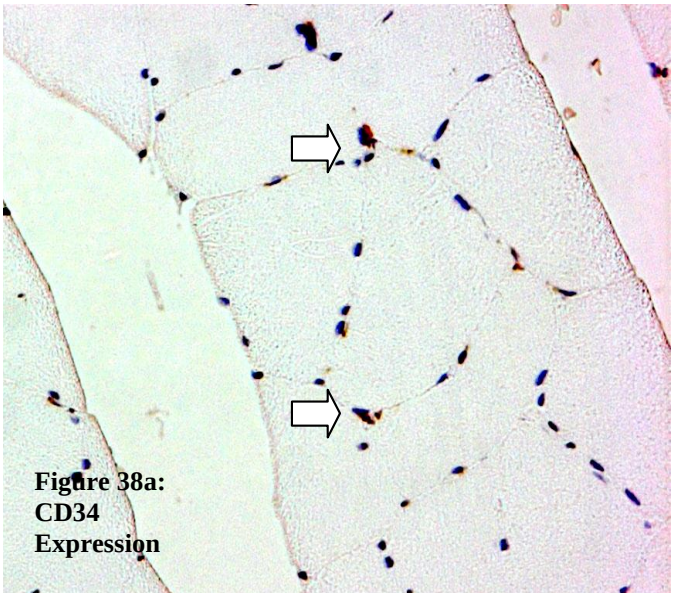


Figure 38a: CD34 Expression

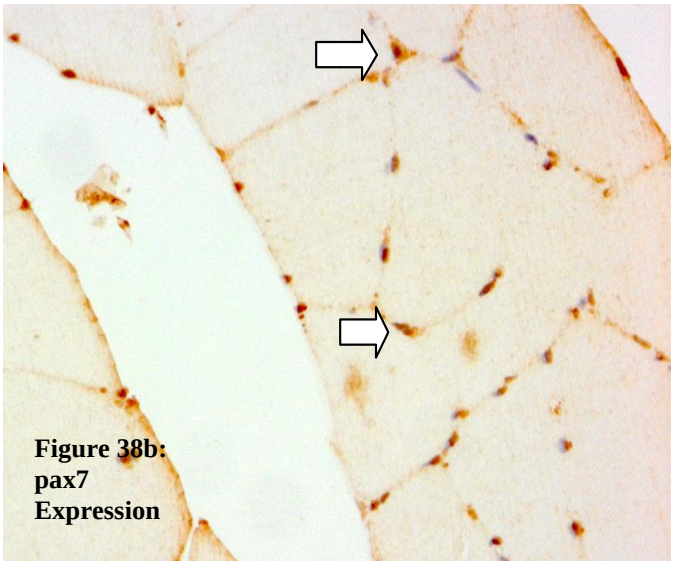


Figure 38b: pax7 Expression

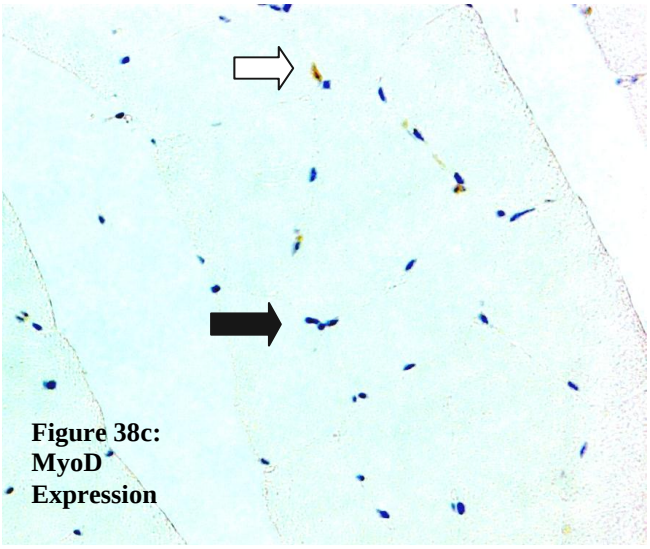


Figure 38: Sequential staining of active and quiescent satellite cells (sample L, section ii); Figure38a: CD34 stains quiescent satellite cells and are indicated by white arrows. Two have been identified in this section at x400 magnification. Figure 38b: pax7 stains all satellite cells, indicated by white arrows. Figure 38c: MyoD stain for active satellite cells, positive cell indicated by a white arrow, negative cell expression of MyoD as would be expected in quiescent SCs (213). A positive result in all three sections was rare and indicates the presence of an active satellite cell. If a cell stained for CD34 and pax7 alone, it indicates a quiescent satellite cell.

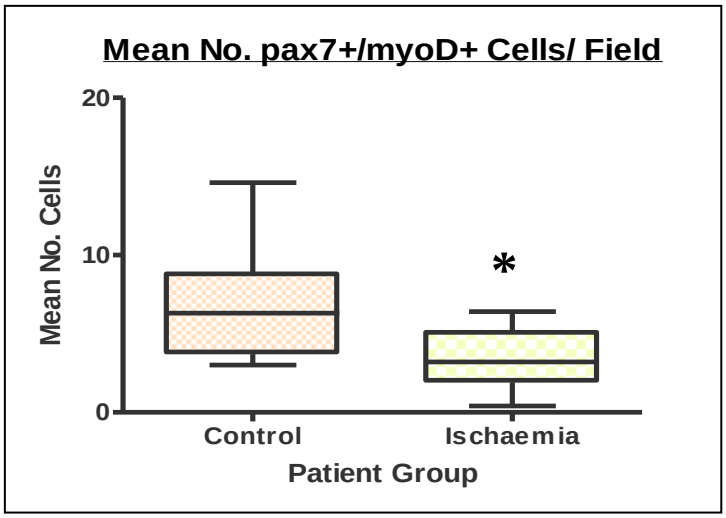
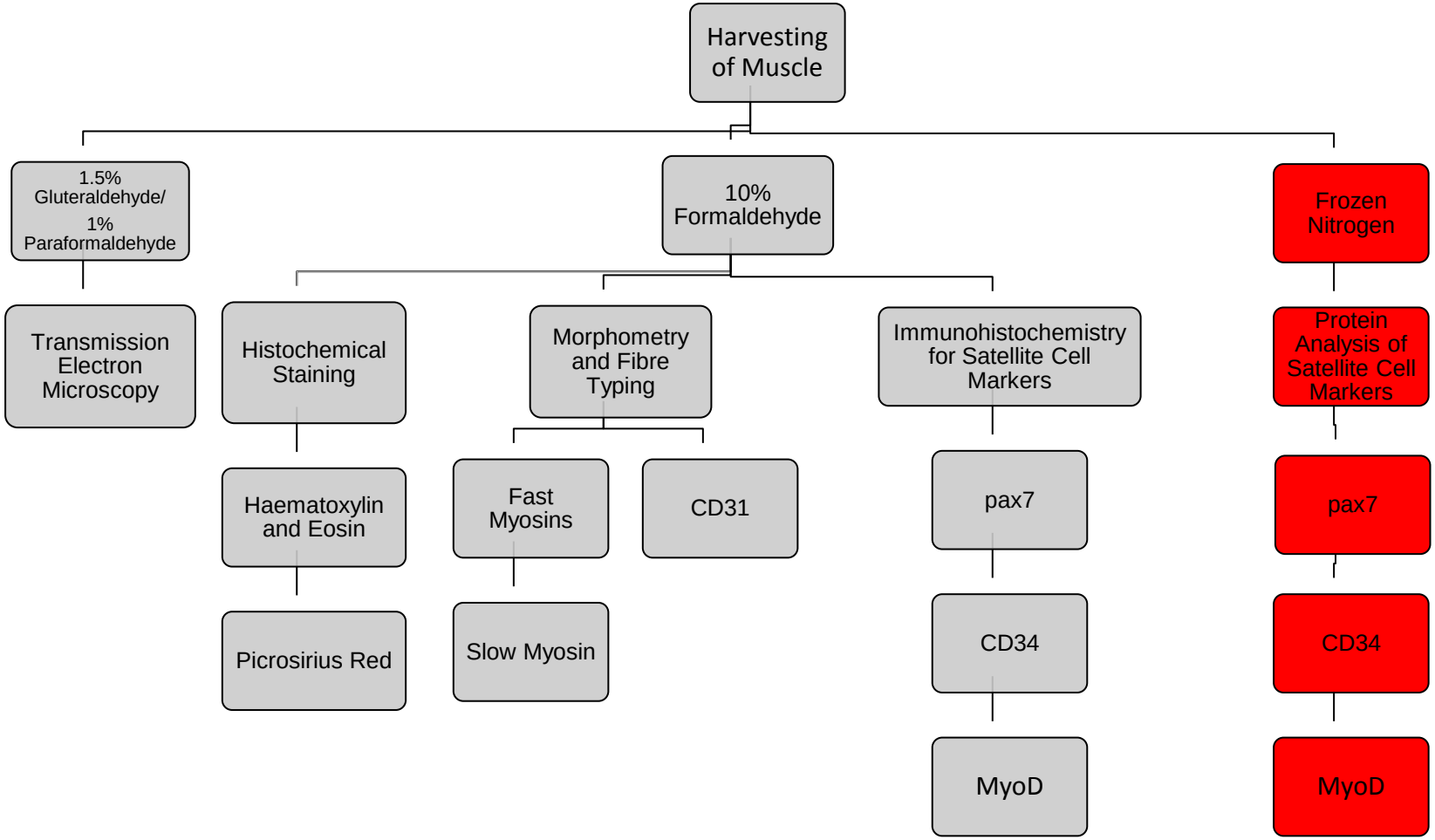


Figure 39: Mean number of pax7+/MyoD+ cells seen per 400 x 300µms field at x200 magnification In comparison, ischaemic sections displayed significantly less active satellite cells than control tissue (*p=0.0089; CI=95%). There was a x2.08 fold decrease in the number of pax7+/MyoD+ cells.

4.4.6 Figure 40: Protein analysis of satellite cell markers via western blotting



4.4.7 Protein analysis of satellite cell markers via western blotting

Nuclear extracts were used to confirm the elevated chromagen levels visualised by immunohistochemistry in the samples analysed previously.

4.4.8 CD34 and pax7 levels are significantly raised in ischaemic adult human skeletal muscle, indicating greater numbers of quiescent SCs

To try and quantify the differences in expression of CD34, pax7 and MyoD protein analysis via Western Blot was performed and both pax7 and CD34 were found to be significantly raised while MyoD was significantly decreased in CLI. This also verified immunohistochemistry and electron microscopy observations**.** Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control for the samples and was found to be comparable throughout the samples as shown below. Lanes 1-6 were control samples, 7-12 were ischaemic.

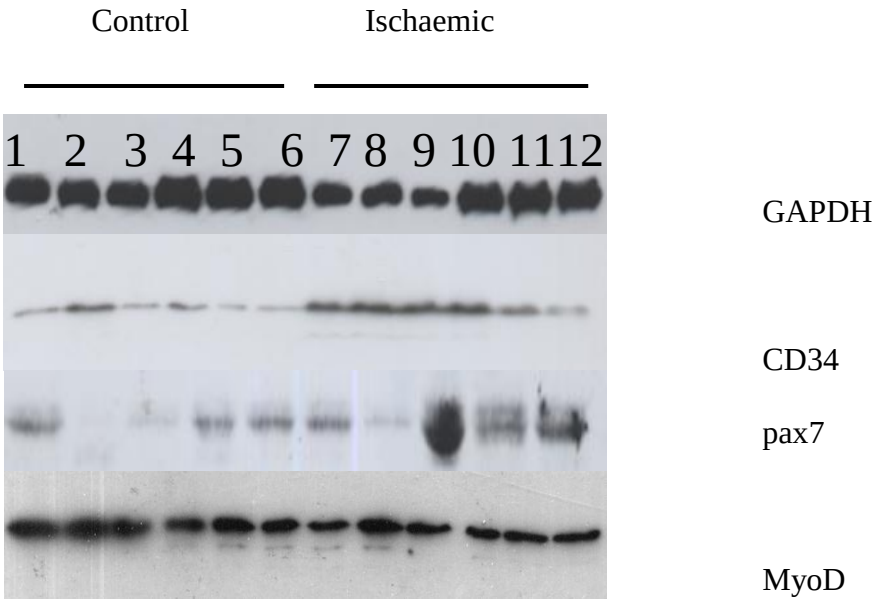


Figure 41: Densitometry of Satellite Cell Markers:

To determine CD34 expression, Western blots of the samples were performed as described previously. There was a significantly stronger expression observed from the CLI group tissue (7-12) than the control group samples (1-6) at the expected molecular weight of CD34, or 116kDa.

The molecular weight of MyoD is 45kD. There was no significant difference in MyoD expression in Control tissue when compared to Ischaemic tissue. A clean band was observed in both experiments when repeated.

The molecular weight of Pax7 is 57 kDa. It can be seen that the pax7 band is expressed strongly in the ischaemic samples and less so in the control tissue at the expected molecular weight. This was confirmed by relative densitometry as a nine fold increase. This experiment was repeated twice

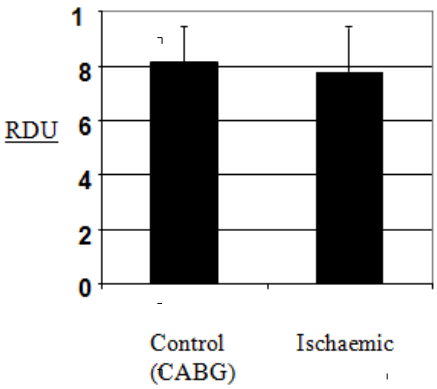


Figure 42a: MyoD Protein Expression Is Similar in CLI

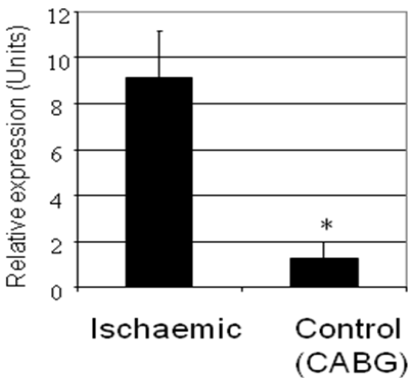


Figure 42b: pax7 Protein Expression Is nine fold higher in CLI *p<0.05)

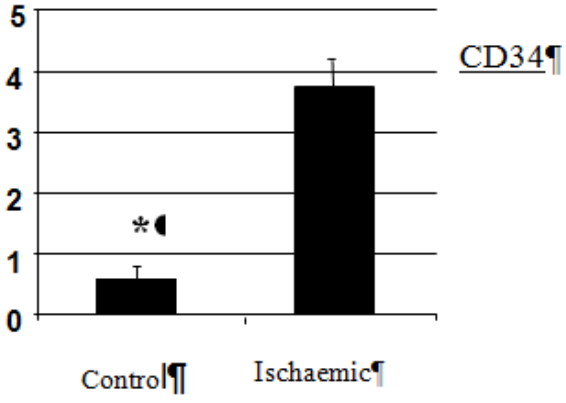


Figure 42c: Expression of CD34 in ischaemia is raised significantly, a x2 fold increase over control tissue; (p<0.05)

4.4.9 Pax7 Elevation


Ischaemic

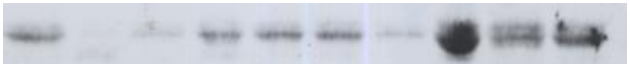

Control

Figure 43: Pax7 Expression is clearly greater in pax7 as shown here in Ischaemic muscle and as shown earlier in RDUs. There is a nine fold increase in pax7 in CLI. ($p<0.05$)

4.4.10 Summary of satellite cell findings

- The muscle response to ischaemia is an attempt to overcome adverse conditions but the myogenic response, like the angiogenic response, never fully matures.
- CD34 a marker of HSCs and quiescent satellite cells is significantly elevated in CLI
- Pax7+CD34+MyoD- mark quiescent satellite cells and these were significantly increased in Critically Ischaemic Human Adult Skeletal Muscle.
- Pax7 a transcription factor necessary for the myogenic fate of satellite cell progeny is elevated in CLI as shown by immunohistochemistry and protein analysis via Western Blot (a nine fold increase) and it may be over expression of this inhibiting differentiation of myoblasts and the activation of satellite cells as has been postulated by other authors.
- MyoD expression in satellite cells is significantly reduced in staining frequency, but in protein analysis, due to its presence in large amounts in cell sarcoplasm, there was no quantifiable difference in protein expression overall.
- Pax7+MyoD+ positive or active satellite cells were significantly less abundant in CLI

Chapter 5 Discussion

5.1 Morphology and Morphometry of Skeletal Muscle in CLI

This study generally concurs with previous studies that show ischaemic muscle displays specific pathognomonic changes. Great disorder is apparent in CLI. This disordered architecture is not only seen in ischaemic myopathy but also in conditions such as Duchenne muscular dystrophy (DMD) which affects innervation as demonstrated in DMD, **see Figure 44**. The variability in fibre size is an indication of ongoing regeneration and degeneration is also typical of the muscular dystrophies (150). Interestingly in both CLI and DMD, there is a well reported increase in fibrofatty tissue and the number of type II fibres is markedly reduced and those type II fibres left actually have a much larger cross sectional area (CSA) than type I fibres (214).

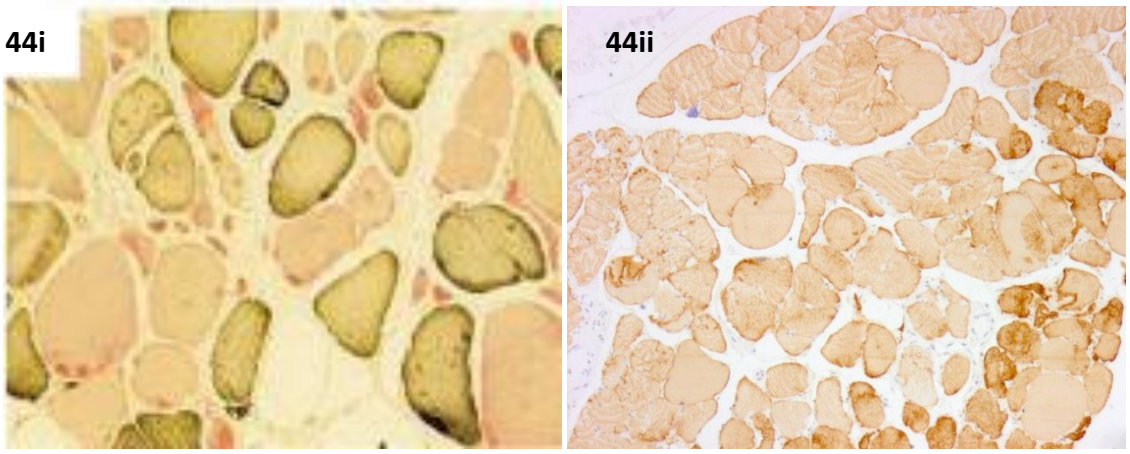


Figure 44i: Comparison of Duchenne Muscular Dystrophy (adapted *from Behan (215)* to 44ii: Ischaemic Muscle

As may be expected, both conditions display some morphology in common. Small rounded fibres can be seen with increased fibre size diversity, resulting from atrophy of myofibres and the more dynamic process of myoblast differentiation and maturation into newly formed myotubes.

The muscle fibres display plasticity through apoptosis and fibre type shift and in this way remodel to the conditions required of them. While the end organ atrophies and undergoes apoptosis to reduce its energy requirements, the capillary network supplying it inversely increases, demonstrating such remodelling is an active process (200). Some fibre types are more resistant to atrophy and ischaemia than others and in this study there was a reduction in fibre CSA of around x 1.5 fold, in line with previous findings (198;200). Even taking this atrophy into account, myonuclei per fibre ratio was not found to significantly differ in either between fibre types nor after exposure to CLI and this concurs with previous studies that have suggested that both satellite cells (SCs) and myonuclei have a domain of influence which remains stable despite injury or disease (88). In younger adults, the regenerative activity of SCs is well regulated, but this tight co-ordination breaks down in advancing disease and individual satellite cells may act independently of normal cues in advancing (107). Regenerating fibres have been described frequently and are small with central nuclei (65;118;216). In this study, there was no significant difference observed between numbers of centrally occurring nuclei in either the CLI or control groups and this may suggest that the process of regeneration occurs at the same frequency despite the severity of ischaemia.

5.2 Fibre Type and Plasticity

It has been established that the human genome codes for four myosin heavy chain genes, but the fibre type IIb is not expressed in humans as it is in lower order mammals. In the rat, which has the most characterised skeletal muscle, IIb is an important fast twitch muscle and confers a "relatively fast stride frequency" in small mammals, being present even in proportionally slow twitch muscles such as soleus (196). Some confusion arises due to the historical mislabelling of mammalian type IIb MHC which has a similar reactivity to human IIx and was identified as such until relatively recently (62;177;185).

Historically ATPase enzyme histochemistry has been used to assess cross sections to identify fibre types in muscle and the correlation between ATPase and Myosin Heavy Chains is well described (175). However, ATPase histochemistry does not allow for hybrid fibres or those undergoing active plasticity (217) and most studies did not examine samples longitudinally which may have identified a dynamic process of changing MHCs along a single fibre length. Only small studies have performed comparisons between IHC and ATPase techniques but immunohistochemistry is as accurate in differentiating type fibres I from II and may also show better correlation for type IIx than ATPase histochemistry. ATPase is unsuitable for staining fixed formalin tissues due to enzymatic degradation and also has well reported problems in reproducibility due to variations in temperature, pH and incubation time as well as the degradation of stained samples over relatively short periods of time (64;218). IHC lends itself well to the analysis of both fixed formalin and frozen muscle, however as mentioned above, commercially available antibodies are at present unable to reliably distinguish between those fibres that contain IIa/x. Serial sections are required to identify these hybrid fibres, they can only be seen in frozen muscle and only by exclusion.

Similarly, there is no antibody which solely stains MHC IIx in paraffin embedded human tissue. Smerdu used the BF-35 antibody to distinguish IIx and IIa in canine gracilis muscle (185). The same group later showed in frozen rat and human tissues that some MHC type II antibodies displayed a strong signal when reacting with MHC type IIa, yet a weak signal was exhibited by MHC IIc and IIx and on these grounds made a distinction between type IIa and IIx positively staining fibres (57). The fast myosin antibody BF-35 stains ALL MHCs BUT IIx in frozen muscle and identifying the phenotype via sequential slides. Unfortunately, this antibody was not reliable in paraffin fixed human skeletal muscle sections **(see Appendix B).** Therefore the IIa/x hybrid fibre types are currently almost indistinguishable from IIa or IIx fibres. With no microscopic features to distinguish between these two fibre types, IIa and IIa/IIx, the latter can confound results and it is difficult to be sure how many of these fibres are present and how they respond to ischaemia (185).

Type I fibres are thought to be protected from free radicals by higher concentrations of antioxidants (219) but are also thought to be more vulnerable to ischaemic injury as they have a predominantly oxidative metabolism. Type II fibres are innervated by large motor neurones which are more readily affected by ischaemia (144) but other studies suggest they may also be relatively well protected due to their anaerobic metabolism of glycogen (220). This study concurs with the body of evidence that suggests IIx fibres are the most susceptible to CLI.

Previous work into the morphometry type II muscles of the elderly (hamstrings, vastus lateralis, gluteus and tensor fascia lata) demonstrated a decrease in the numbers of all fibre types, with a corresponding size and fibre diameter decrease (144). Type II fibres in this study into ischaemia were also seen to diminish, but as a much faster rate than if age alone had been the causative factor. There was no statistical difference between the ages of the

cohorts. Possibly these fibres are more susceptible to ischaemic damage because of their large neurones or their anaerobic metabolism of glycogen (118;119;138). In ischaemia, the number and proportion of type I fibres either remain the same or even increase (221). Regensteiner et al. (119) noted no significant change in type I fibres, either in number, size or average area in the calf muscles of 26 adult humans with CLI compared to those examined in 6 control patients. They found that none of the fibres examined displayed a significant size or number difference, but type II fibres did appear reduced in their average area coverage throughout the muscle. This preferential atrophy of type II fibres concurs with other reports (60;65;68;118;119;222).

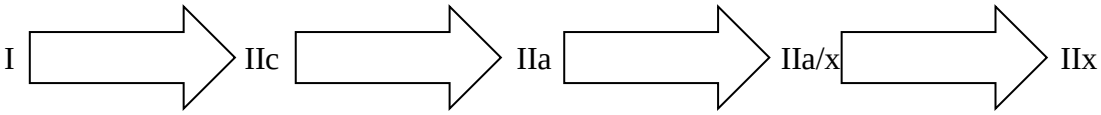
Other authors do not describe significant relative changes in type II MHCs (60;65;118;119;172). Williamson et al. found that in resistance training, hybrid fibres are in fact reduced (223). In this study, chronic ischaemia led to a significant increase in hybrid fibres, despite similar conditions to resistance training being replicated in the tissues. However, the ischaemia observed in resistance exercise is intermittent and therefore muscle respond in a different way to constant ischaemia.

Previous work has shown that resident satellite cells form type II fibres in normal muscle. The perceived increase in type I fibre proportion may indicate that while the larger type II fibres are preferentially lost, CLI may stimulate SCs to form type I fibres instead. There may also be an increase in fibre type shift, with greater flux from type II fibres to type I fibres. (101;224).

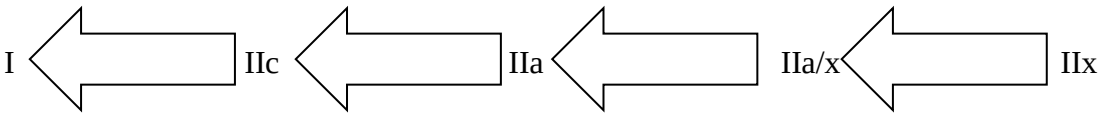
All fibre types showed a significant reduction in CSA, except for the hybrid IIc fibres, which were not only significantly more numerous in number in the CLI patients (x2.4 fold increase,

p=0.0147; 95% CI) but also were the only group of muscle fibres not to display significant atrophy. Type IIx fibres were preferentially lost in ischaemic muscle, with a significant x4.67 fold reduction in fibre proportion (p=0.0039). They also displayed the greatest cross sectional area atrophy. The morphology and morphometric findings of this study have been outlined and compared to previous work in Table 11 below:

The diagram below illustrates the relative fibre shift caused by various pathological and physiological conditions including the fibre type shift of this study which highlight the preferential shift to type I fibres which suggests these fibres are more resistant to ischaemia. Such increased activity as displayed by regeneration of fibres, fibre type shift and increased hybrid fibres indicates the dynamic process of plasticity.



Denervation(124), Inactivity (221;225-229), Low-Gravity (114) COAD (187;188;230) Sprinters/ Weightlifters (221), Intermittent Ischaemia (229)



Critical Limb Ischaemia (60;98;231;232), Endurance Exercise (44;62;62;221), Age (228;233-235)

Figure 45: Plasticity of Myofibrils: fibre type shift as governed by physiological or pathological condition

	Hart et al.	Hedberg et al. [20]	Sjostrom (65)	Regensteiner (119)	McGuigan (172)	Askew(60)	Albani(118)
	Human Ischaemic Tissue Gastroc 10 CLI vs 10 Con	Human Ischaemic Tissue Gastroc 5 CLI vs 5 Con	Human Ischaemic Ant Tibial 22 CLI	Human Ischaemic Calf Muscles 26 CLI vs 6 Con	Human Ischaemic Gastroc 14 CLI vs 8 Con	Human Ischaemic Gastroc 16 CLI vs 13 Con	Human Ischaemic Tissue Ant Tibial 9 CLI
Fibre Size Diversity	Increased	Increased	Increased	N/A	N/A	Increased	Increased
Fibre Type I Diameter	↓	↔	↔	↔	↓	↓	↓/↔
Fibre Type II Diameter	↓	↓	↔	↓	↓	↓	↓
Total Fibre Number	↑	↓	↔	↔	N/A	↓	N/A
No. Fibre Type I	↑	↑	↔	↔	↑	↑	↑
No. Fibre Type II	↓	↓	↔	↔	a: ↔ x: ↓	a: ↓ x: ↔	↔
Fibre Type I (%) area	N/A	N/A	↔	↔	↓	↓	↔
Fibre Type II (%) area	N/A	N/A	↔	↓ Atrophy	↓	↓	↔
Connective Tissue	↑	↑	↑ (30%)	↔	N/A	N/A	N/A
Angular Fibres	↑	↑	↑	↑	N/A	N/A	↑
Central Nu	↔	N/A	↑	N/A	N/A	N/A	↑
Fibre Type Clumping	↑	↑	↑	↑	N/A	N/A	↑
C:F Ratio	↑	N/A	N/A	N/A	↑ (23%)	↓ (20-25%)	N/A

Table 11: Morphometric differences between healthy and ischaemic human skeletal muscle [A Review of Previous Findings].
CLI: Limb Ischaemia (varying severity) C:F Capillary to Fibre Ratio
Con: Healthy Controls (not age-matched in all cases)

5.3 Satellite cell number and behaviour in CLI

There has been little data published on the numbers and activity of satellite cells in response to ischaemia. The co-ordinated action of these cells is required for the process of plasticity in the form of pre-conditioning, remodelling of muscle through fibre shift and the efficient repair of damaged myofibres. This resident group of bipotent stem cells and have shown themselves to be capable of self renewal or myoblast, myonucleus or myotubes formation (205). It has been suggested that the renewal of the satellite cell population may result from the increased number of circulating haemopoietic cells derived from bone marrow or blood (158;162;163). There was an increase in the number of circulating bone marrow derived haematopoietic stem cells (HSCs) which reliably express CD34 (186).

These cells are implicated in the process of angiogenesis in CLI skeletal muscle as well as the repair and regeneration of muscle by replenishing the satellite cell population. SCs have been transplanted into the heart with a regeneration of cardiomyocytes seen around them (83;106;159;236). The possibility remains that under the certain conditions, with the correct transcription factors they may be encouraged to form any mesodermal tissue, given that they are or can be stimulated to become multipotent cells. Circulating Bone Marrow derived HSCs expressing CD34 were certainly observed in this study to be much more numerous in ischaemic muscle (x2 fold increase; $p<0.0001$, 95% CI), this was also demonstrated via protein analysis ($p=0.05$).

Pax7 is a transcription factor expressed by most SCs, active and quiescent; it was consistently raised in CLI muscle as demonstrated through morphometric analysis, immunohistochemistry and protein analysis. Immunohistochemistry and morphometric analysis demonstrated x2.4

fold increase in pax7 positive nuclei. In control muscle, there was a relatively clean and specific chromagen signal, however in ischaemic muscle, there was generalised over expression of pax7 throughout the samples. This may be as a result of ectopic expression in the tissues although some authors believe that there is little expression of pax7 except from satellite cells or perhaps myonuclei "in distress" (73;213). Others report that pax7 may be expressed by immature satellite-cell derived myoblasts (237). Protein analysis demonstrated a significant x9 fold increase ($p=0.05$).

Pax7 delays the expression of myogenin for terminal differentiation, does not actually prevent the latter nor does it promote the quiescent state (237). While many satellite cells continued to express both pax7 and MyoD, some stop expressing the latter and withdraw to a quiescent state. Pax7 in vitro causes increased self-renewal of satellite cells and reduced myogenic progression and differentiation (116). However, in the environment of CLI, over expression of pax7 may well have a further effect on the MRFs, inhibiting their action.

Due to the increased activity seen in the morphometric analysis of the CLI muscle, an increase in fibre numbers per field, regenerating fibres and hybrid fibres in ischaemia, it was hypothesised that the distribution of MyoD as marker of activated satellite cells would be increased accordingly (210). Interestingly, MyoD positive nuclei were not increased in ischaemic muscle, but showed a significant decrease from control samples ($p<0.001$) perhaps indicative of the reduction in fast twitch fibres. Also, significantly fewer cells (x2.1 fold decrease) co stained positive for pax7 and myoD on sequential slides. This is a novel finding that would infer that activated satellite cells are significantly reduced in number in ischaemic tissue and this may be due to over-expression of pax7 (148). Zammit et al. (2006) had

observed in pax7-null C2C12 cells undergoing normal myogenesis that ectopic pax7 could stimulate MyoD expression and was able to act both up and downstream of MyoD.

Previously it has been demonstrated that myogenin levels are higher in slow-twitch muscles, while MyoD is associated with fast-type fibres and it has been proposed that myogenin and MyoD may function as intermediaries to selectively regulate fibre-type-dependent contractile gene expression. Specifically, because myogenin controls expression of a battery of muscle-specific genes, including slow MHC expression (238), MyoD may control fast twitch fibres and myogenin control slow MHC gene expression (175). Consequently, an increase in the myogenin to MyoD ratio would preferentially drive expression of slow-type contractile proteins in skeletal muscle.

Active satellite cells, those expressing pax7 and MyoD have been shown in previous studies to be more abundant in oxidative muscle compared to glycolytic muscle. Indeed the composition of muscle is crucial to its response to the MRFs and satellite cell activity (73;239). However, in this study, there was no obvious correlation between fibre type and the presence of SCs. There was also a pattern of cytoplasmic staining with MyoD which represents myofibre differentiation. Protein analysis of MyoD did not show a significant difference expression between control or CLI tissue

Pax7 and CD34 expression determines the presence of a quiescent satellite or myogenic precursor cells. These have the ability to form either myofibres or self renew and are greatly increased in ischaemia but exist in an inactive state and have stopped expressing MyoD. Local SCs are probably the only significant source of new myoblasts in the adult tissue, but the contribution of bone-derived haematopoietic cells, vascular progenitor cells, or interstitial

cells has not been fully evaluated (213). Analysis of these cells in sequential slides revealed a significantly higher frequency of quiescent satellite cells observed in the CLI specimens (x2.9 fold increase; $p<0.0001$: 95% CI). Due to the increased numbers of SCs seen in ischaemic skeletal muscle, it is clear that the exhaustion of satellite cell mitogenesis is not the primary cause for failed regeneration but more their failure to mature and differentiate. This appears to be the case in other chronic illnesses such as Duchenne muscular dystrophy (212).

Active SCs assessed through the use of anti- MyoD and pax7 antibodies were sparsely present and may represent a small isolated sub population which is responsible for renewing the satellite cell pool (148). These active SCs may be derived from local skeletal tissue or from circulating haematopoietic cells that become committed to a myogenic fate due to pax7 present. It is unclear as yet whether all satellite cells are capable of self renewal or this is a function of an undiscovered subset (73;148), which are similar to mitotically quiescent satellite cells and that do not express myogenic differentiation factor or pax7. Some have postulated these constitute a group of cells responsible for replenishing the satellite cell pool (148;240;241). Yoshida et al 1998 named these “reserve cells” (242), Rudnicki labelled them “side population” cells (243).

Therapeutic potential arises from the extraordinary ability of skeletal muscle to repair and regenerate. It is clear that the resident population of quiescent satellite cells or a circulating haematopoietic stem cell population that can be recruited to assist in the processes of myogenesis or angiogenesis. Indeed in ischaemia, both of these processes actually show an increased frenetic activity, but often disordered and unable to achieve maturity. Stem cells may have the potential to repair all mesodermal tissue either once stimulated in native muscle or by invasive introduction and help provide an answer to remodelling or preconditioning

human skeletal muscle. Satellite cells may be capable of differentiating into multiple mesodermal tissues and the selective inhibition of pax7 and the MRFs may yield a different fate for these cells and allow them to contribute to angiogenesis or even neurogenesis (9).

5.4 Limitations of the study

There are areas of limitation with this study, not least that it is observational and not interventional. The number of patients within each cohort numbered only 10 in number due to accessibility to tissue, patient choice and patient suitability for the study. The tissue samples were carefully vetted for architectural integrity and nec rotic samples as discussed below were deemed unsuitable.

In addition to the CLI and the Control (CABG) groups there was a role for further cohorts to be analysed, in particular completely fit and healthy patients or those with less severe peripheral disease, i.e. those with intermittent claudication. Risk factors were comparable across both groups but diabetics were included in both groups and these may suffer from a different disease process than atherosclerotics (16).

Following a cohort of cardiac patients prospectively until they displayed PAD symptomology would have been ideal to gain an understanding of how the increasing impact of atherosclerosis affects muscle and satellite cell (SC) structure and function during disease progression. This was impossible due to time constraints of the thesis, the massive number of patients that would have to be recruited and the repeated biopsies required and the accumulated risk of complications. Percutaneous biopsies would have been one alternative

solution but these yield small samples and are quite painful and inconvenient for the patients. A further cohort of patients would have been useful for this study, those with no global atherosclerosis. Although these patients are not comparable in risk factors or pathology, they would have allowed comparison to “normal” tissue and levels of stem cells and antibodies.

Proliferation markers could have been used to confirm the suitability of samples and affirm proliferation of muscle tissue or satellite cells. However, the presence of positively staining sarcoplasmic MyoD and nuclear pax7 through immunohistochemistry confirmed that active processes were occurring within harvested tissue. Any harvested tissue that displayed the features of necrosis such as loss of nuclei, loss of normal surface membrane integrity and loss of polygonal architecture was excluded from the study. At the time of harvesting, it would have been useful to have collected snap frozen tissue, prepared it with OCT and use those antibodies not suitable for paraffin fixed tissue analysis, such as the embryonic myosin or BF-35.

The use of such preservation would have allowed the verification of type IIx fibres and possibly the identification of IIa/x hybrid fibres but the same fields could not have been analysed. Although paraffin embedded samples display superior preservation of muscle architecture, this process of preservation precludes the use of many antibodies. The inability of the study to correctly identify IIa/x hybrid fibres means that there is some confounding of data. It is unclear just how numerous these hybrid fibres are in ischaemic (or even normal) muscle and may be present in significant numbers. Indeed the hybrid IIc fibres were not thought to be numerous for many years.

There were minor, but very time consuming, problems with reproducibility in that slides were sequential and therefore, in most immunohistochemistry experiments, at least 5 viable areas were required on each sequential slide, a sequence numbering eight in total. In particular, areas of analysis in the larger biopsy samples were difficult to identify and morphometric analysis of these was also extremely time consuming. Due to artefact and the required precision of measurements, automating this process was just not viable (192;215).

Western Blotting depends on the specificity of an antibody to recognise an epitope. Due to the use of tissue rather than cultured cells, the achieved chemiluminescent signal can be affected by non specific binding of other proteins causing false positives. Protein expression could have been augmented with RT PCR to assess RNA and therefore gene expression.

5.5 Future Work

Ischaemia as a driving force for fibre type change can be difficult to separate from other co existing factors. It can be safely assumed that those with symptomatic disease of chronic disease have a more sedentary life style. Those with angina or heart failure similarly have reduced exercise tolerance, however this was not objectively measured in this study. The shift towards type II fibres due to inactivity (221) is at odds with that seen due to increasing age or ischaemia. This could potentially confuse data collection, but using two cohorts with such similar demographics negates the effects of age and the overall effect of CLI on adult human skeletal muscle seems to indicate that type I fibres are least affected by worsening ischaemia and there appears to be a preferential loss of type II myofibres in CLI. Perhaps because of these difficulties, previous studies vary in their findings regarding which fibres are the most

resilient in the face of advancing ischaemia, certainly they all agree that the regenerative capacity of muscle becomes exhausted with worsening disease (83;92;108;233;244). It is important to highlight that this study, unlike those that have gone before, has used a comparable population in demographics and comorbidities. There may be less variation in results than if the control group used was perfectly healthy. The fact these populations were comparable allows a better understanding of the timeline of pathognomonic and fibre type changes that occur in the advanced stages of atherosclerosis.

Homogeneity in muscle morphometry research is difficult to achieve, due to the variety and complexity of normal skeletal muscle structure and function, but meta-analysis of current experimental data is difficult. In human tissue certainly, more work on single muscles, such as gastrocnemius or perhaps the biceps femoris would certainly help elucidate the processes of repair and regeneration and allow for manipulation to improve CLI outcomes. All muscle groups differ in their myosin composition and this may alter with age and other co-morbidities. Samples from groups with similar age, sex, past medical history, medications and smoking history will also reduce the amount of variables that may confound data (94). Patient selection based on the severity of symptoms including exercise tolerance would be useful, as it is becoming clear that changes are linked with *clearly defined stages of disease* (60)

In data collection and interpretation, both cross-sectional and longitudinal biopsies both yield useful information required for detailed analysis. Behan et al. used 80 fibres per sample (215) and Lexell and Taylor suggested that a minimum number of 25 myofibres are assessed per biopsy, and that single biopsies are to be avoided if possible (otherwise a minimum of 100

should be analysed for cross sectional area (CSA) and other morphometric measurements (189;190).

Further, improvements to SDS-Page techniques originally suggested by Talmadge and further improved by Bammam, have clarified the interpretation of data by adequately separating the MHC isoforms IIa and IIx (245;246). Electrophoretic methods indicate an overall shift in MHC type from type II fibres to type I fibres, but exact results are still obscured by the presence of hybrid or jump fibres, particularly IIa/x. More data regarding the comparative reliability of all of the above experimental techniques is also required.

Commercially available antibodies for both frozen and paraffin embedded tissue would be helpful to specifically identify the MHC composition of any given fibre and allow a more accurate assessment of its response to a given environmental stimulus (101). There is currently no single marker that will universally recognise all satellite cells, active or quiescent, nor able to differentiate between stem cell populations and consensus needs to be reached upon the simplest solution to this problem. There is evidence to both support and refute the notion that circulating bone marrow derived stem cells (247) are the source of satellite cells and/ or endothelial cells (158;248) and work continues to try and elucidate the origin of satellite cells and whether they themselves are responsible for self renewal, circulating bone derived haematopoietic stem cells or a combination of both.

Isolation of satellite cells from muscle or even C2C12 cell cultures and then subjecting them to hypoxia with the addition of various angiogenic or myogenic mediators will help elucidate the pathways required to form ischaemia resistant muscle. It may be more revealing to inhibit combinations of myogenic committing factors such as the MRFs or pax7 preventing their

action on SCs and their precursors. It is already known resident SCs can be induced to form cardiomyocytes and neural lineages (106;159;236;249).

Perhaps there exists a mechanism to recruit or even inject these pluripotent mesodermal cells to provide a therapeutic option which can promote a collateral circulation, reinnervate tissue, activate satellite cells and improve muscle composition, thereby reversing the effects of atherosclerosis and CLI (250;251). Indeed, the harnessing of satellite cells and their multipotent mesodermal potential may hold the answer in the treatment of this devastating disease.

Chapter 6 Conclusion

Human Critical Limb Ischaemia (CLI) is associated with an increase in cell regeneration and remodelling with an increase in myofibres, myofibre plasticity, angiogenesis and proliferation of satellite cells. There is a marked alteration in muscle morphology in CLI compared to asymptomatic atherosclerosis.

Both type I and type II fibre types atrophy in the face of CLI. Type I fibres are more numerous and seem to be the least susceptible in the face of overwhelming chronic ischaemia. The greater proportion of type I fibres through the preferential atrophy of type II fibres, in CLI leads to the perceived fibre type shift. However, more type IIc hybrid fibres are present in greater degrees of ischaemia, indicating active plasticity of myofibres and this is the only identified group of fibres seemingly resistant to atrophy.

There is an abundance of satellite cells in CLI, confirmed by electron microscopy. This is also apparent through the use of markers such as pax7 and CD34. There is massive over expression of pax7 in CLI and even when co localised with CD34, a marker of haematopoietic stem cells, there is still a dramatic increase in the number of positively staining nuclei, indicating an increase in quiescent satellite cells. Those satellite cells also expressing MyoD and thus activated satellite cells, become more scarce in ischaemic tissue. Over expression of pax7 may account for this, down regulating activation of satellite cells and leaving them in a dormant state. It is possible that the insufficient response of satellite cells in the CLI environment is in part due to this overexpression.

The results of this study suggest an incomplete response to ischaemia and an attempt at adaptation by both the angiogenic and myogenic pathways which is insufficient to meet the demands of tissue repair and regeneration. It is perhaps premature to base large assumptions on such a small study, but there is evidence that larger observational and interventional studies are required to elucidate further the role of pax7 and the possibility that overexpression of pax7 has an inhibitory effect on stem cells of all types.

Reference List

(1) Murabito JM, Evans JC, Nieto K, Larson MG, Levy D, Wilson PW. Prevalence and clinical correlates of peripheral arterial disease in the Framingham Offspring Study. Am Heart J 2002 Jun;143(6):961-5.

(2) Kannel WB. Risk stratification in hypertension: new insights from the Framingham Study. Am J Hypertens 2000 Jan;13(1 Pt 2):3S-10S.

(3) Pasternak RC, Criqui MH, Benjamin EJ, Fowkes FG, Isselbacher EM, McCullough PA, et al. Atherosclerotic Vascular Disease Conference: Writing Group I: epidemiology. Circulation 2004 Jun 1;109(21):2605-12.

(4) Norgren L, Hiatt WR, Dormandy JA, Nehler MR, Harris KA, Fowkes FG, et al. Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II). Eur J Vasc Endovasc Surg 2007;33 Suppl 1:S1-75.

(5) Dormandy JA, Rutherford RB. Management of peripheral arterial disease (PAD). TASC Working Group. TransAtlantic Inter-Society Consensus (TASC). J Vasc Surg 2000 Jan;31(1 Pt 2):S1-S296.

(6) Tierney S, Fennessy F, Hayes DB. ABC of arterial and vascular disease. Secondary prevention of peripheral vascular disease. BMJ 2000 May 6;320(7244):1262-5.

(7) Gottsater A. Managing risk factors for atherosclerosis in critical limb ischaemia. Eur J Vasc Endovasc Surg 2006 Nov;32(5):478-83.

(8) Moxey PW, Hofman D, Hinchliffe RJ, Jones K, Thompson MM, Holt PJ. Epidemiological study of lower limb amputation in England between 2003 and 2008. Br J Surg 2010 Sep;97(9):1348-53.

(9) Shireman PK. The chemokine system in arteriogenesis and hind limb ischemia. J Vasc Surg 2007 Jun;45 Suppl A:A48-A56.

(10) Franks PJ, Morgan PA. Health-related quality of life with chronic leg ulceration
3. Expert Rev Pharmacoecon Outcomes Res 2003 Oct;3(5):611-22.

(11) Posnett J, Franks PJ. The burden of chronic wounds in the UK
1. Nurs Times 2008 Jan 22;104(3):44-5.

(12) Gordois A, Scuffham P, Shearer A, Oglesby A, Tobian JA. The health care costs of diabetic peripheral neuropathy in the US
5. Diabetes Care 2003 Jun;26(6):1790-5.

(13) Bashir R, Cooper CJ. Evaluation and medical treatment of peripheral arterial disease
16. Curr Opin Cardiol 2003 Nov;18(6):436-43.

(14) Hart W.M., Guest J.F. Critical limb ischaemia; the burden of illness in the UK. Br Med Econ 1995;8:211-21.

(15) Carson R, Waugh N. Comparison of the acute costs of lower limb bypass surgery in diabetics. 1998.

(16) Schroeder TV. The TASC supplement - international recommendations for management of peripheral arterial disease. Eur J Vasc Endovasc Surg 2000 Jun;19(6):563.

(17) Singh S, Evans L, Datta D, Gaines P, Beard JD. The costs of managing lower limb-threatening ischaemia. Eur J Vasc Endovasc Surg 1996 Oct;12(3):359-62.

(18) Office National Statistics. Smoking and Drinking; General Household Survey 2005. 2009 Sep 29.

(19) Smoking Kills; A White Paper on Tobacco, Cm 4177, Secretary of State, Dobson F, Dewer D, Mowlam M, Michael A, (1998).

(20) Faxon DP, Fuster V, Libby P, Beckman JA, Hiatt WR, Thompson RW, et al. Atherosclerotic Vascular Disease Conference: Writing Group III: pathophysiology. Circulation 2004 Jun 1;109(21):2617-25.
(21) Faxon DP, Creager MA, Halperin JL, Bernard DB, Ryan TJ. Redistribution of regional blood flow following angiotensin-converting enzyme inhibition. Comparison of normal subjects and patients with heart failure. Am J Med 1984 May 31;76(5B):104-10.
(22) Creager MA, Jones DW, Easton JD, Halperin JL, Hirsch AT, Matsumoto AH, et al. Atherosclerotic Vascular Disease Conference: Writing Group V: medical decision making and therapy. Circulation 2004 Jun 1;109(21):2634-42.
(23) Feinglass J, Brown JL, LoSasso A, Sohn MW, Manheim LM, Shah SJ, et al. Rates of lower-extremity amputation and arterial reconstruction in the United States, 1979 to 1996. Am J Public Health 1999 Aug;89(8):1222-7.
(24) Singer DR, Kite A. Management of hypertension in peripheral arterial disease: does the choice of drugs matter? Eur J Vasc Endovasc Surg 2008 Jun;35(6):701-8.
(25) Santilli JD, Santilli SM. Chronic critical limb ischemia: diagnosis, treatment and prognosis. Am Fam Physician 1999 Apr 1;59(7):1899-908.
(26) Cavanagh SP, Gough MJ, Homer-Vanniasinkam S. The role of the neutrophil in ischaemia-reperfusion injury: potential therapeutic interventions. Cardiovasc Surg 1998 Apr;6(2):112-8.
(27) Donnelly R, Collinson DJ. Consensus on anti-platelet therapy in peripheral arterial disease: saving lives and limbs
4. Platelets 2003 Nov;14(7-8):403-6.
(28) Risau W. Mechanisms of angiogenesis. Nature 1997 Apr 17;386(6626):671-4.
(29) Bauters C, Asahara T, Zheng LP, Takeshita S, Bunting S, Ferrara N, et al. Site-specific therapeutic angiogenesis after systemic administration of vascular endothelial growth factor. J Vasc Surg 1995 Feb;21(2):314-24.
(30) Rissanen TT, Vajanto I, Yla-Herttuala S. Gene therapy for therapeutic angiogenesis in critically ischaemic lower limb - on the way to the clinic. Eur J Clin Invest 2001 Aug;31(8):651-66.
(31) Brevetti LS, Sarkar R, Chang DS, Ma M, Paek R, Messina LM. Administration of adenoviral vectors induces gangrene in acutely ischemic rat hindlimbs: role of capsid protein-induced inflammation. J Vasc Surg 2001 Sep;34(3):489-96.
(32) Tsurumi Y, Takeshita S, Chen D, Kearney M, Rossow ST, Passeri J, et al. Direct intramuscular gene transfer of naked DNA encoding vascular endothelial growth factor augments collateral development and tissue perfusion. Circulation 1996 Dec 15;94(12):3281-90.
(33) Tsurumi Y, Kearney M, Chen D, Silver M, Takeshita S, Yang J, et al. Treatment of acute limb ischemia by intramuscular injection of vascular endothelial growth factor gene. Circulation 1997 Nov 4;96(9 Suppl):II-8.
(34) Tsurumi Y, Kearney M, Chen D, Silver M, Takeshita S, Yang J, et al. Treatment of acute limb ischemia by intramuscular injection of vascular endothelial growth factor gene
1. Circulation 1997 Nov 4;96(9 Suppl):II-8.
(35) Bashir R, Cooper CJ. Evaluation and medical treatment of peripheral arterial disease. Curr Opin Cardiol 2003 Nov;18(6):436-43.
(36) Collinson DJ, Donnelly R. Therapeutic angiogenesis in peripheral arterial disease: can biotechnology produce an effective collateral circulation? Eur J Vasc Endovasc Surg 2004 Jul;28(1):9-23.

(37) Aviles RJ, Annex BH, Lederman RJ. Testing clinical therapeutic angiogenesis using basic fibroblast growth factor (FGF-2). Br J Pharmacol 2003 Oct;140(4):637-46.
(38) Fowkes FG, Price JF. Gene therapy for critical limb ischaemia: the TAMARIS trial
3. Lancet 2011 Jun 4;377(9781):1894-6.
(39) Timaran CH, Prault TL, Stevens SL, Freeman MB, Goldman MH. Iliac artery stenting versus surgical reconstruction for TASC (TransAtlantic Inter-Society Consensus) type B and type C iliac lesions. J Vasc Surg 2003 Aug;38(2):272-8.
(40) Johnston KW. Iliac arteries: reanalysis of results of balloon angioplasty. Radiology 1993 Jan;186(1):207-12.
(41) Lee ES, Steenson CC, Trimble KE, Caldwell MP, Kuskowski MA, Santilli SM. Comparing patency rates between external iliac and common iliac artery stents. J Vasc Surg 2000 May;31(5):889-94.
(42) Hirsch AT, Haskal ZJ, Hertzer NR, Bakal CW, Creager MA, Halperin JL, et al. ACC/AHA 2005 Practice Guidelines for the management of patients with peripheral arterial disease (lower extremity, renal, mesenteric, and abdominal aortic): a collaborative report from the American Association for Vascular Surgery/Society for Vascular Surgery, Society for Cardiovascular Angiography and Interventions, Society for Vascular Medicine and Biology, Society of Interventional Radiology, and the ACC/AHA Task Force on Practice Guidelines (Writing Committee to Develop Guidelines for the Management of Patients With Peripheral Arterial Disease): endorsed by the American Association of Cardiovascular and Pulmonary Rehabilitation; National Heart, Lung, and Blood Institute; Society for Vascular Nursing; TransAtlantic Inter-Society Consensus; and Vascular Disease Foundation. Circulation 2006 Mar 21;113(11):e463-e654.
(43) Veith FJ, Gupta SK, Ascer E, White-Flores S, Samson RH, Scher LA, et al. Six-year prospective multicenter randomized comparison of autologous saphenous vein and expanded polytetrafluoroethylene grafts in infrainguinal arterial reconstructions. J Vasc Surg 1986 Jan;3(1):104-14.
(44) Nasr MK, McCarthy RJ, Budd JS, Horrocks M. Infrainguinal bypass graft patency and limb salvage rates in critical limb ischemia: influence of the mode of presentation
1. Ann Vasc Surg 2003 Mar;17(2):192-7.
(45) Evans WE, Hayes JP, Vermilion BD. Effect of a failed distal reconstruction on the level of amputation. Am J Surg 1990 Aug;160(2):217-20.
(46) Creager MA, Cooke JP, Mendelsohn ME, Gallagher SJ, Coleman SM, Loscalzo J, et al. Impaired vasodilation of forearm resistance vessels in hypercholesterolemic humans. J Clin Invest 1990 Jul;86(1):228-34.
(47) Celermajer DS, Sorensen KE, Georgakopoulos D, Bull C, Thomas O, Robinson J, et al. Cigarette smoking is associated with dose-related and potentially reversible impairment of endothelium-dependent dilation in healthy young adults. Circulation 1993 Nov;88(5 Pt 1):2149-55.
(48) Loscalzo J. Nitric oxide insufficiency, platelet activation, and arterial thrombosis. Circ Res 2001 Apr 27;88(8):756-62.
(49) Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. Circulation 2002 Mar 5;105(9):1135-43.
(50) Lubos E, Handy DE, Loscalzo J. Role of oxidative stress and nitric oxide in atherothrombosis. Front Biosci 2008;13:5323-44.
(51) Werner N, Nickenig G. Influence of cardiovascular risk factors on endothelial progenitor cells: limitations for therapy? Arterioscler Thromb Vasc Biol 2006 Feb;26(2):257-66.

(52) Dandona P, Dhindsa S, Ghanim H, Chaudhuri A. Angiotensin II and inflammation: the effect of angiotensin-converting enzyme inhibition and angiotensin II receptor blockade. J Hum Hypertens 2007 Jan;21(1):20-7.
(53) Libby P. Inflammation in atherosclerosis. Nature 2002 Dec 19;420(6917):868-74.
(54) Faxon DP, Fuster V, Libby P, Beckman JA, Hiatt WR, Thompson RW, et al. Atherosclerotic Vascular Disease Conference: Writing Group III: pathophysiology. Circulation 2004 Jun 1;109(21):2617-25.
(55) Arbanas J, Klasan GS, Nikolic M, Jerkovic R, Miljanovic I, Malnar D. Fibre type composition of the human psoas major muscle with regard to the level of its origin. J Anat 2009 Dec;215(6):636-41.
(56) Rogers A. Cells and Tissues; An Introduction to Histology and Cell Biology. Academic Press; 1983. p. 116-32.
(57) Smerdu V, Soukup T. Demonstration of myosin heavy chain isoforms in rat and humans: the specificity of seven available monoclonal antibodies used in immunohistochemical and immunoblotting methods. Eur J Histochem 2008 Jul;52(3):179-90.
(58) BULLER AJ, ECCLES JC, ECCLES RM. Interactions between motoneurones and muscles in respect of the characteristic speeds of their responses
1. J Physiol 1960 Feb;150:417-39.
(59) BULLER AJ, ECCLES JC, ECCLES RM. Differentiation of fast and slow muscles in the cat hind limb
2. J Physiol 1960 Feb;150:399-416.
(60) Askew CD, Green S, Walker PJ, Kerr GK, Green AA, Williams AD, et al. Skeletal muscle phenotype is associated with exercise tolerance in patients with peripheral arterial disease. J Vasc Surg 2005 May;41(5):802-7.
(61) Bojsen-Moller J, Hansen P, Aagaard P, Svantesson U, Kjaer M, Magnusson SP. Differential displacement of the human soleus and medial gastrocnemius aponeuroses during isometric plantar flexor contractions in vivo
1. J Appl Physiol 2004 Nov;97(5):1908-14.
(62) Dahmane R, Djordjevic S, Smerdu V. Adaptive potential of human biceps femoris muscle demonstrated by histochemical, immunohistochemical and mechanomyographical methods. Med Biol Eng Comput 2006 Nov;44(11):999-1006.
(63) Edgerton VR, Barnard RJ, Peter JB, Maier PA, Simpson DR. Properties of immobilized hind-limb muscles of the Galago senegalensis
1. Exp Neurol 1975 Jan;46(1):115-31.
(64) Behan WM, Cossar DW, Madden HA, McKay IC. Validation of a simple, rapid, and economical technique for distinguishing type 1 and 2 fibres in fixed and frozen skeletal muscle. J Clin Pathol 2002 May;55(5):375-80.
(65) Sjostrom M, Angquist KA, Rais O. Intermittent claudication and muscle fiber fine structure: correlation between clinical and morphological data. Ultrastruct Pathol 1980 Jul;1(3):309-26.
(66) Erzen I, Cvetko E, Obreza S, Angaut-Petit D. Fiber types in the mouse levator auris longus muscle: a convenient preparation to study muscle and nerve plasticity. J Neurosci Res 2000 Mar 1;59(5):692-7.
(67) Erzen I. Plasticity of Skeletal muscle studied by stereology. Image Analysis Stereology 2004;23:143-52.
(68) Andersen JL. Muscle fibre type adaptation in the elderly human muscle. Scand J Med Sci Sports 2003 Feb;13(1):40-7.

(69) Mauro A. Satellite Cell of Skeletal Muscle Fibers. Journal of Biophysical and Biochemical Cytology 1961;9(2):493-&.
(70) Schmalbruch H, Hellhammer U. The number of satellite cells in normal human muscle. Anat Rec 1976 Jul;185(3):279-87.
(71) Watkins SC, Cullen MJ. A quantitative study of myonuclear and satellite cell nuclear size in Duchenne's muscular dystrophy, polymyositis and normal human skeletal muscle. Anat Rec 1988 Sep;222(1):6-11.
(72) Allbrook D. Skeletal muscle regeneration. Muscle Nerve 1981 May;4(3):234-45.
(73) Enns DL, Tiidus PM. Estrogen influences satellite cell activation and proliferation following downhill running in rats. J Appl Physiol 2008 Feb;104(2):347-53.
(74) Kurek JB, Bower JJ, Romanella M, Koentgen F, Murphy M, Austin L. The role of leukemia inhibitory factor in skeletal muscle regeneration. Muscle Nerve 1997 Jul;20(7):815-22.
(75) Creuzet S, Lescaudron L, Li Z, Fontaine-Perus J. MyoD, myogenin, and desmin-nls-lacZ transgene emphasize the distinct patterns of satellite cell activation in growth and regeneration. Exp Cell Res 1998 Sep 15;243(2):241-53.
(76) Garry DJ, Meeson A, Elterman J, Zhao Y, Yang P, Bassel-Duby R, et al. Myogenic stem cell function is impaired in mice lacking the forkhead/winged helix protein MNF. Proc Natl Acad Sci U S A 2000 May 9;97(10):5416-21.
(77) Rosenblatt JD, Yong D, Parry DJ. Satellite cell activity is required for hypertrophy of overloaded adult rat muscle. Muscle Nerve 1994 Jun;17(6):608-13.
(78) Schultz E, McCormick KM. Skeletal muscle satellite cells. Rev Physiol Biochem Pharmacol 1994;123:213-57.
(79) McCormick D, Chong H, Hobbs C, Datta C, Hall PA. Detection of the Ki-67 antigen in fixed and wax-embedded sections with the monoclonal antibody MIB1. Histopathology 1993 Apr;22(4):355-60.
(80) Winchester PK, Gonyea WJ. Regional injury and the terminal differentiation of satellite cells in stretched avian slow tonic muscle. Dev Biol 1992 Jun;151(2):459-72.
(81) Watt DJ, Morgan JE, Clifford MA, Partridge TA. The movement of muscle precursor cells between adjacent regenerating muscles in the mouse. Anat Embryol (Berl) 1987;175(4):527-36.
(82) Gregory M.A., Mars M. Mobilisation of satellite cells following ischaemia and reperfusion in primate skeletal muscle. Sports Medicine 2004;16(1):17-24.
(83) Hawke TJ, Garry DJ. Myogenic satellite cells: physiology to molecular biology. J Appl Physiol 2001 Aug;91(2):534-51.
(84) Rosenblatt JD, Parry DJ. Adaptation of rat extensor digitorum longus muscle to gamma irradiation and overload. Pflugers Arch 1993 May;423(3-4):255-64.
(85) Barton-Davis ER, Shoturma DI, Sweeney HL. Contribution of satellite cells to IGF-I induced hypertrophy of skeletal muscle. Acta Physiol Scand 1999 Dec;167(4):301-5.
(86) Darr KC, Schultz E. Exercise-induced satellite cell activation in growing and mature skeletal muscle. J Appl Physiol 1987 Nov;63(5):1816-21.
(87) Allen DL, Roy RR, Edgerton VR. Myonuclear domains in muscle adaptation and disease. Muscle Nerve 1999 Oct;22(10):1350-60.
(88) Brack AS, Bildsoe H, Hughes SM. Evidence that satellite cell decrement contributes to preferential decline in nuclear number from large fibres during murine age-related muscle atrophy. J Cell Sci 2005 Oct 15;118(Pt 20):4813-21.

(89) Cornelison DD, Wold BJ. Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. Dev Biol 1997 Nov 15;191(2):270-83.
(90) Dedkov EI, Borisov AB, Wernig A, Carlson BM. Aging of skeletal muscle does not affect the response of satellite cells to denervation. J Histochem Cytochem 2003 Jul;51(7):853-63.
(91) Dedkov EI, Kostrominova TY, Borisov AB, Carlson BM. MyoD and myogenin protein expression in skeletal muscles of senile rats. Cell Tissue Res 2003 Mar;311(3):401-16.
(92) Renault V, Rolland E, Thornell LE, Mouly V, Butler-Browne G. Distribution of satellite cells in the human vastus lateralis muscle during aging. Exp Gerontol 2002 Dec;37(12):1513-4.
(93) Sjostrom M, Angquist KA, Rais O. Intermittent claudication and muscle fiber fine structure: correlation between clinical and morphological data
1. Ultrastruct Pathol 1980 Jul;1(3):309-26.
(94) Harris BA. The influence of endurance and resistance exercise on muscle capillarization in the elderly: a review. Acta Physiol Scand 2005 Oct;185(2):89-97.
(95) Darr KC, Schultz E. Exercise-induced satellite cell activation in growing and mature skeletal muscle. J Appl Physiol 1987 Nov;63(5):1816-21.
(96) Zammit PS, Heslop L, Hudon V, Rosenblatt JD, Tajbakhsh S, Buckingham ME, et al. Kinetics of myoblast proliferation show that resident satellite cells are competent to fully regenerate skeletal muscle fibers. Exp Cell Res 2002 Nov 15;281(1):39-49.
(97) Schmalbruch H, Hellhammer U. The number of satellite cells in normal human muscle. Anat Rec 1976 Jul;185(3):279-87.
(98) Hedberg B, Angquist KA, Henriksson-Larsen K, Sjostrom M. Fibre loss and distribution in skeletal muscle from patients with severe peripheral arterial insufficiency. Eur J Vasc Surg 1989 Aug;3(4):315-22.
(99) Havenith MG, Visser R, Schrijvers-van Schendel JM, Bosman FT. Muscle fiber typing in routinely processed skeletal muscle with monoclonal antibodies. Histochemistry 1990;93(5):497-9.
(100) Hikida RS, Van NS, Murray JD, Staron RS, Gordon SE, Kraemer WJ. Myonuclear loss in atrophied soleus muscle fibers. Anat Rec 1997 Mar;247(3):350-4.
(101) Sajko S, Kubinova L, Cvetko E, Kreft M, Wernig A, Erzen I. Frequency of M-cadherin-stained satellite cells declines in human muscles during aging. J Histochem Cytochem 2004 Feb;52(2):179-85.
(102) Roth SM, Martel GF, Ivey FM, Lemmer JT, Metter EJ, Hurley BF, et al. Skeletal muscle satellite cell populations in healthy young and older men and women. Anat Rec 2000 Dec 1;260(4):351-8.
(103) Renault V, Piron-Hamelin G, Forestier C, DiDonna S, Decary S, Hentati F, et al. Skeletal muscle regeneration and the mitotic clock. Exp Gerontol 2000 Sep;35(6-7):711-9.
(104) Dreyer HC, Blanco CE, Sattler FR, Schroeder ET, Wiswell RA. Satellite cell numbers in young and older men 24 hours after eccentric exercise. Muscle Nerve 2006 Feb;33(2):242-53.
(105) Renault V, Thornell LE, Eriksson PO, Butler-Browne G, Mouly V. Regenerative potential of human skeletal muscle during aging. Aging Cell 2002 Dec;1(2):132-9.
(106) Wei Y, Li Y, Chen C, Stoelzel K, Kaufmann AM, Albers AE. Human skeletal muscle-derived stem cells retain stem cell properties after expansion in myosphere culture. Exp Cell Res 2011 Apr 15;317(7):1016-27.

(107) Dedkov EI, Borisov AB, Wernig A, Carlson BM. Aging of skeletal muscle does not affect the response of satellite cells to denervation. J Histochem Cytochem 2003 Jul;51(7):853-63.
(108) Renault V, Thornell LE, Eriksson PO, Butler-Browne G, Mouly V. Regenerative potential of human skeletal muscle during aging. Aging Cell 2002 Dec;1(2):132-9.
(109) Kitzmann M, Carnac G, Vandromme M, Primig M, Lamb NJ, Fernandez A. The muscle regulatory factors MyoD and myf-5 undergo distinct cell cycle-specific expression in muscle cells. J Cell Biol 1998 Sep 21;142(6):1447-59.
(110) Kitzmann M, Vandromme M, Schaeffer V, Carnac G, Labbe JC, Lamb N, et al. cdk1- and cdk2-mediated phosphorylation of MyoD Ser200 in growing C2 myoblasts: role in modulating MyoD half-life and myogenic activity. Mol Cell Biol 1999 Apr;19(4):3167-76.
(111) Lassar AB, Skapek SX, Novitch B. Regulatory mechanisms that coordinate skeletal muscle differentiation and cell cycle withdrawal. Curr Opin Cell Biol 1994 Dec;6(6):788-94.
(112) Mal A, Sturniolo M, Schiltz RL, Ghosh MK, Harter ML. A role for histone deacetylase HDAC1 in modulating the transcriptional activity of MyoD: inhibition of the myogenic program. EMBO J 2001 Apr 2;20(7):1739-53.
(113) Valdez MR, Richardson JA, Klein WH, Olson EN. Failure of Myf5 to support myogenic differentiation without myogenin, MyoD, and MRF4. Dev Biol 2000 Mar 15;219(2):287-98.
(114) Bodine-Fowler SC, Pierotti DJ, Talmadge RJ. Functional and cellular adaptation to weightlessness in primates. J Gravit Physiol 1995;2(1):43-6.
(115) Zammit PS, Carvajal JJ, Golding JP, Morgan JE, Summerbell D, Zolnerciks J, et al. Myf5 expression in satellite cells and spindles in adult muscle is controlled by separate genetic elements. Dev Biol 2004 Sep 15;273(2):454-65.
(116) McFarlane C, Hennebry A, Thomas M, Plummer E, Ling N, Sharma M, et al. Myostatin signals through Pax7 to regulate satellite cell self-renewal
1. Exp Cell Res 2008 Jan 15;314(2):317-29.
(117) Russell B, Dix DJ, Haller DL, Jacobs-El J. Repair of injured skeletal muscle: a molecular approach. Med Sci Sports Exerc 1992 Feb;24(2):189-96.
(118) Albani M, Megalopoulos A, Kiskinis D, Parashos SA, Grigoriadis N, Guiba-Tziampiri O. Morphological, histochemical, and interstitial pressure changes in the tibialis anterior muscle before and after aortofemoral bypass in patients with peripheral arterial occlusive disease. BMC Musculoskelet Disord 2002;3:8.
(119) Regensteiner JG, Wolfel EE, Brass EP, Carry MR, Ringel SP, Hargarten ME, et al. Chronic changes in skeletal muscle histology and function in peripheral arterial disease. Circulation 1993 Feb;87(2):413-21.
(120) Hedberg B, Angquist KA, Henriksson-Larsen K, Sjostrom M. Fibre loss and distribution in skeletal muscle from patients with severe peripheral arterial insufficiency. Eur J Vasc Surg 1989 Aug;3(4):315-22.
(121) Pette D, Sketelj J, Skorjanc D, Leisner E, Traub I, Bajrovic F. Partial fast-to-slow conversion of regenerating rat fast-twitch muscle by chronic low-frequency stimulation. J Muscle Res Cell Motil 2002;23(3):215-21.
(122) Te KG, Reggiani C. Skeletal muscle fibre type specification during embryonic development. J Muscle Res Cell Motil 2002;23(1):65-9.
(123) Morgan JE, Partridge TA. Muscle satellite cells. Int J Biochem Cell Biol 2003 Aug;35(8):1151-6.

(124) Dedkov EI, Kostrominova TY, Borisov AB, Carlson BM. Reparative myogenesis in long-term denervated skeletal muscles of adult rats results in a reduction of the satellite cell population. Anat Rec 2001 Jun 1;263(2):139-54.
(125) Oustanina S, Hause G, Braun T. Pax7 directs postnatal renewal and propagation of myogenic satellite cells but not their specification
1. EMBO J 2004 Aug 18;23(16):3430-9.
(126) Rodeheffer MS. Tipping the scale: muscle versus fat. Nat Cell Biol 2010 Feb;12(2):102-4.
(127) Naveiras O, Nardi V, Wenzel PL, Hauschka PV, Fahey F, Daley GQ. Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment. Nature 2009 Jul 9;460(7252):259-63.
(128) Hill M, Wernig A, Goldspink G. Muscle satellite (stem) cell activation during local tissue injury and repair. J Anat 2003 Jul;203(1):89-99.
(129) Joe AW, Yi L, Natarajan A, Le GF, So L, Wang J, et al. Muscle injury activates resident fibro/adipogenic progenitors that facilitate myogenesis. Nat Cell Biol 2010 Feb;12(2):153-63.
(130) Arnold L, Henry A, Poron F, Baba-Amer Y, van RN, Plonquet A, et al. Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. J Exp Med 2007 May 14;204(5):1057-69.
(131) Tidball JG, Villalta SA. Regulatory interactions between muscle and the immune system during muscle regeneration. Am J Physiol Regul Integr Comp Physiol 2010 May;298(5):R1173-R1187.
(132) Tidball JG. Inflammatory processes in muscle injury and repair
7. Am J Physiol Regul Integr Comp Physiol 2005 Feb;288(2):R345-R353.
(133) Arnold L, Henry A, Poron F, Baba-Amer Y, van RN, Plonquet A, et al. Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. J Exp Med 2007 May 14;204(5):1057-69.
(134) Di CA, De MR, Martelli F, Pompilio G, Capogrossi MC, Germani A. Hypoxia inhibits myogenic differentiation through accelerated MyoD degradation. J Biol Chem 2004 Apr 16;279(16):16332-8.
(135) de la Serna IL, Roy K, Carlson KA, Imbalzano AN. MyoD can induce cell cycle arrest but not muscle differentiation in the presence of dominant negative SWI/SNF chromatin remodeling enzymes. J Biol Chem 2001 Nov 2;276(44):41486-91.
(136) Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: role of the HIF system. Nat Med 2003 Jun;9(6):677-84.
(137) Pette D, Staron RS. Mammalian skeletal muscle fiber type transitions. Int Rev Cytol 1997;170:143-223.
(138) Steinacker JM, Opitz-Gress A, Baur S, Lormes W, Bolkart K, Sunder-Plassmann L, et al. Expression of myosin heavy chain isoforms in skeletal muscle of patients with peripheral arterial occlusive disease. J Vasc Surg 2000 Mar;31(3):443-9.
(139) Dodd SL, Vrabas IS, Stetson DS. Effects of intermittent ischemia on contractile properties and myosin isoforms of skeletal muscle. Med Sci Sports Exerc 1998 Jun;30(6):850-5.
(140) Andersen JL, Gruschy-Knudsen T, Sandri C, Larsson L, Schiaffino S. Bed rest increases the amount of mismatched fibers in human skeletal muscle. J Appl Physiol 1999 Feb;86(2):455-60.
(141) Jennekens FG, Tomlinson BE, Walton JN. Histochemical aspects of five limb muscles in old age. An autopsy study. J Neurol Sci 1971 Nov;14(3):259-76.

(142) McDermott MM, Guralnik JM, Albay M, Bandinelli S, Miniati B, Ferrucci L. Impairments of muscles and nerves associated with peripheral arterial disease and their relationship with lower extremity functioning: the InCHIANTI Study. J Am Geriatr Soc 2004 Mar;52(3):405-10.
(143) Croall DE, DeMartino GN. Calcium-activated neutral protease (calpain) system: structure, function, and regulation
2. Physiol Rev 1991 Jul;71(3):813-47.
(144) Lee WS, Cheung WH, Qin L, Tang N, Leung KS. Age-associated decrease of type IIA/B human skeletal muscle fibers. Clin Orthop Relat Res 2006 Sep;450:231-7.
(145) Lundberg IE. The physiology of inflammatory myopathies: an overview. Acta Physiol Scand 2001 Mar;171(3):207-13.
(146) Lundberg IE. The physiology of inflammatory myopathies: an overview
4. Acta Physiol Scand 2001 Mar;171(3):207-13.
(147) Smith CA, Stauber F, Waters C, Alway SE, Stauber WT. Transforming growth factor-beta following skeletal muscle strain injury in rats
1. J Appl Physiol 2007 Feb;102(2):755-61.
(148) Olguin HC, Olwin BB. Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal. Dev Biol 2004 Nov 15;275(2):375-88.
(149) Beggs ML, Nagarajan R, Taylor-Jones JM, Nolen G, Macnicol M, Peterson CA. Alterations in the TGFbeta signaling pathway in myogenic progenitors with age. Aging Cell 2004 Dec;3(6):353-61.
(150) Cassano M, Quattrocelli M, Crippa S, Perini I, Ronzoni F, Sampaolesi M. Cellular mechanisms and local progenitor activation to regulate skeletal muscle mass. J Muscle Res Cell Motil 2009 Dec;30(7-8):243-53.
(151) Amthor H, Otto A, Vulin A, Rochat A, Dumonceaux J, Garcia L, et al. Muscle hypertrophy driven by myostatin blockade does not require stem/precursor-cell activity
1. Proc Natl Acad Sci U S A 2009 May 5;106(18):7479-84.
(152) Shen W, Li Y, Tang Y, Cummins J, Huard J. NS-398, a cyclooxygenase-2-specific inhibitor, delays skeletal muscle healing by decreasing regeneration and promoting fibrosis. Am J Pathol 2005 Oct;167(4):1105-17.
(153) Sakuma K, Watanabe K, Sano M, Kitajima S, Sakamoto K, Uramoto I, et al. The adaptive response of transforming growth factor-beta 2 and -beta RII in the overloaded, regenerating and denervated muscles of rats. Acta Neuropathol (Berl) 2000 Feb;99(2):177-85.
(154) Risau W. Mechanisms of angiogenesis. Nature 1997 Apr 17;386(6626):671-4.
(155) Tonini T, Rossi F, Claudio PP. Molecular basis of angiogenesis and cancer. Oncogene 2003 Sep 29;22(42):6549-56.
(156) Semenza GL. Angiogenesis in ischemic and neoplastic disorders. Annu Rev Med 2003;54:17-28.
(157) Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature 2000 Sep 14;407(6801):249-57.
(158) Makin AJ, Blann AD, Chung NA, Silverman SH, Lip GY. Assessment of endothelial damage in atherosclerotic vascular disease by quantification of circulating endothelial cells. Relationship with von Willebrand factor and tissue factor. Eur Heart J 2004 Mar;25(5):371-6.
(159) Arsic N, Mamaeva D, Lamb NJ, Fernandez A. Muscle-derived stem cells isolated as non-adherent population give rise to cardiac, skeletal muscle and neural lineages. Exp Cell Res 2008 Apr 1;314(6):1266-80.

(160) Pagani FD, DerSimonian H, Zawadzka A, Wetzel K, Edge AS, Jacoby DB, et al. Autologous skeletal myoblasts transplanted to ischemia-damaged myocardium in humans. Histological analysis of cell survival and differentiation. J Am Coll Cardiol 2003 Mar 5;41(5):879-88.
(161) Gustafsson T, Knutsson A, Puntschart A, Kaijser L, Nordqvist AC, Sundberg CJ, et al. Increased expression of vascular endothelial growth factor in human skeletal muscle in response to short-term one-legged exercise training. Pflugers Arch 2002 Sep;444(6):752-9.
(162) Tuomisto TT, Rissanen TT, Vajanto I, Korkeela A, Rutanen J, Yla-Herttuala S. HIF-VEGF-VEGFR-2, TNF-alpha and IGF pathways are upregulated in critical human skeletal muscle ischemia as studied with DNA array. Atherosclerosis 2004 May;174(1):111-20.
(163) Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med 2003 Jun;9(6):669-76.
(164) Rissanen TT, Markkanen JE, Arve K, Rutanen J, Kettunen MI, Vajanto I, et al. Fibroblast growth factor 4 induces vascular permeability, angiogenesis and arteriogenesis in a rabbit hindlimb ischemia model. FASEB J 2003 Jan;17(1):100-2.
(165) Jones PF. Tied up (or down?) with angiopoietins. Angiogenesis 1997;1(1):38-44.
(166) Rissanen TT, Vajanto I, Hiltunen MO, Rutanen J, Kettunen MI, Niemi M, et al. Expression of vascular endothelial growth factor and vascular endothelial growth factor receptor-2 (KDR/Flk-1) in ischemic skeletal muscle and its regeneration. Am J Pathol 2002 Apr;160(4):1393-403.
(167) Palmer-Kazen U, Wariaro D, Luo F, Wahlberg E. Vascular endothelial cell growth factor and fibroblast growth factor 2 expression in patients with critical limb ischemia. J Vasc Surg 2004 Mar;39(3):621-8.
(168) Wang L, Kwak JH, Kim SI, He Y, Choi ME. Transforming growth factor-beta1 stimulates vascular endothelial growth factor 164 via mitogen-activated protein kinase kinase 3-p38alpha and p38delta mitogen-activated protein kinase-dependent pathway in murine mesangial cells. J Biol Chem 2004 Aug 6;279(32):33213-9.
(169) Ho TK, Rajkumar V, Ponticos M, Leoni P, Black DC, Abraham DJ, et al. Increased endogenous angiogenic response and hypoxia-inducible factor-1alpha in human critical limb ischemia. J Vasc Surg 2006 Jan;43(1):125-33.
(170) Darland DC, D'Amore PA. Blood vessel maturation: vascular development comes of age. J Clin Invest 1999 Jan;103(2):157-8.
(171) Ho TK, Rajkumar V, Black CM, Abraham DJ, Baker DM. Increased angiogenic response but deficient arteriolization and abnormal microvessel ultrastructure in critical leg ischaemia. Br J Surg 2006 Nov;93(11):1368-76.
(172) McGuigan MR, Bronks R, Newton RU, Sharman MJ, Graham JC, Cody DV, et al. Muscle fiber characteristics in patients with peripheral arterial disease. Med Sci Sports Exerc 2001 Dec;33(12):2016-21.
(173) Carry MR, Ringel SP, Starcevich JM. Distribution of Capillaries in Normal and Diseased Human Skeletal-Muscle. Muscle & Nerve 1986 Jun;9(5):445-54.
(174) Ho TK, Rajkumar V, Ponticos M, Leoni P, Black DC, Abraham DJ, et al. Increased endogenous angiogenic response and hypoxia-inducible factor-1alpha in human critical limb ischemia. J Vasc Surg 2006 Jan;43(1):125-33.
(175) Hughes SM, Cho M, Karsch-Mizrachi I, Travis M, Silberstein L, Leinwand LA, et al. Three slow myosin heavy chains sequentially expressed in developing mammalian skeletal muscle. Dev Biol 1993 Jul;158(1):183-99.

(176) Fazeli S, Wells DJ, Hobbs C, Walsh FS. Altered secondary myogenesis in transgenic animals expressing the neural cell adhesion molecule under the control of a skeletal muscle alpha-actin promoter. J Cell Biol 1996 Oct;135(1):241-51.
(177) Smerdu V, Karsch-Mizrachi I, Campione M, Leinwand L, Schiaffino S. Type IIx myosin heavy chain transcripts are expressed in type IIb fibers of human skeletal muscle. Am J Physiol 1994 Dec;267(6 Pt 1):C1723-C1728.
(178) Shorey CD, Cleland KW. Problems associated with the morphometric measurement of transverse skeletal muscle fibers: I. Analysis of frozen sections
1. Anat Rec 1983 Nov;207(3):523-31.
(179) Caplin M, Savage K, Khan K, Brett B, Rode J, Varro A, et al. Expression and processing of gastrin in pancreatic adenocarcinoma. Br J Surg 2000 Aug;87(8):1035-40.
(180) Dammeijer PF, van MH, van DP, Moorman AF, Habets P, Manni JJ, et al. Stapedius muscle fibre composition in the rat. Hear Res 2000 Mar;141(1-2):169-79.
(181) Gosker HR, Engelen MP, van MH, van Dijk PJ, van der Vusse GJ, Wouters EF, et al. Muscle fiber type IIX atrophy is involved in the loss of fat-free mass in chronic obstructive pulmonary disease. Am J Clin Nutr 2002 Jul;76(1):113-9.
(182) Junqueira LC, Bignolas G, Brentani RR. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections
2. Histochem J 1979 Jul;11(4):447-55.
(183) Whittaker P KRBDPJ. Quantitative Assessment of myocardial collagen with picrosirius red staining and circularly polarized light. Basic Research In Cardiology 1994.
(184) McCormick D, Chong H, Hobbs C, Datta C, Hall PA. Detection of the Ki-67 antigen in fixed and wax-embedded sections with the monoclonal antibody MIB1. Histopathology 2002 Sep;41(3A):173-8.
(185) Smerdu V, Strbenc M, Meznaric-Petrusa M, Fazarinc G. Identification of myosin heavy chain I, IIa and IIx in canine skeletal muscles by an electrophoretic and immunoblotting study. Cells Tissues Organs 2005;180(2):106-16.
(186) Czeiger D, Dukhno O, Douvdevani A, Porat Y, Shimoni D, Fulga V, et al. Transient Extremity Ischemia Augments CD34+ Progenitor Cell Availability. Stem Cell Rev 2011 Sep;7(3):639-45.
(187) Whittom F, Jobin J, Simard PM, LeBlanc P, Simard C, Bernard S, et al. Histochemical and morphological characteristics of the vastus lateralis muscle in patients with chronic obstructive pulmonary disease. Med Sci Sports Exerc 1998 Oct;30(10):1467-74.
(188) Franssen FM, Wouters EF, Schols AM. The contribution of starvation, deconditioning and ageing to the observed alterations in peripheral skeletal muscle in chronic organ diseases. Clin Nutr 2002 Feb;21(1):1-14.
(189) Lexell J, Taylor C, Sjostrom M. Analysis of sampling errors in biopsy techniques using data from whole muscle cross sections. J Appl Physiol 1985 Oct;59(4):1228-35.
(190) Lexell J, Taylor CC. Variability in muscle fibre areas in whole human quadriceps muscle: how to reduce sampling errors in biopsy techniques. Clin Physiol 1989 Aug;9(4):333-43.
(191) Sant'ana Pereira JA, Wessels A, Nijtmans L, Moorman AF, Sargeant AJ. New method for the accurate characterization of single human skeletal muscle fibres demonstrates a relation between mATPase and MyHC expression in pure and hybrid fibre types. J Muscle Res Cell Motil 1995 Feb;16(1):21-34.

(192) Slavin G, Sowter C, Ward P, Paton K. Measurement of striated muscle fibre diameters using interactive computer-aided microscopy. J Clin Pathol 1982 Nov;35(11):1268-71.
(193) Behan WM, Cossar DW, Madden HA, McKay IC. 102236963
Validation of a simple, rapid, and economical technique for distinguishing type 1 and 2 fibres in fixed and frozen skeletal muscle. J Clin Pathol 2002 May;55(5):375-80.
(194) Cho M, Hughes SM, Karsch-Mizrachi I, Travis M, Leinwand LA, Blau HM. Fast myosin heavy chains expressed in secondary mammalian muscle fibers at the time of their inception. J Cell Sci 1994 Sep;107 (Pt 9):2361-71.
(195) Dammeijer PF, van MH, van DP, Moorman AF, Habets P, Manni JJ, et al. Stapedius muscle fibre composition in the rat. Hear Res 2000 Mar;141(1-2):169-79.
(196) Schiaffino S, Gorza L, Sartore S, Saggin L, Ausoni S, Vianello M, et al. Three myosin heavy chain isoforms in type 2 skeletal muscle fibres. J Muscle Res Cell Motil 1989 Jun;10(3):197-205.
(197) Anvar MD, Khiabani HZ, Nesland JM, Stranden E, Kroese AJ. Vascular and stromal features in the skin of the lower limb in patients with chronic critical limb ischaemia (CLI) and oedema
2. Eur J Vasc Endovasc Surg 2000 Aug;20(2):125-31.
(198) Hoppeler H, Vogt M, Weibel ER, Fluck M. Response of skeletal muscle mitochondria to hypoxia. Exp Physiol 2003 Jan;88(1):109-19.
(199) Brunk UT, Terman A. The mitochondrial-lysosomal axis theory of aging: accumulation of damaged mitochondria as a result of imperfect autophagocytosis. Eur J Biochem 2002 Apr;269(8):1996-2002.
(200) Hoppeler H, Kleinert E, Schlegel C, Claassen H, Howald H, Kayar SR, et al. Morphological adaptations of human skeletal muscle to chronic hypoxia. Int J Sports Med 1990 Feb;11 Suppl 1:S3-S9.
(201) Nanobashvili J, Neumayer C, Fugl A, Punz A, Blumer R, Prager M, et al. Ischemia/reperfusion injury of skeletal muscle: plasma taurine as a measure of tissue damage
2. Surgery 2003 Jan;133(1):91-100.
(202) Sajko S, Kubinova L, Cvetko E, Kreft M, Wernig A, Erzen I. Frequency of M-cadherin-stained satellite cells declines in human muscles during aging. J Histochem Cytochem 2004 Feb;52(2):179-85.
(203) Allen DL, Roy RR, Edgerton VR. Myonuclear domains in muscle adaptation and disease. Muscle Nerve 1999 Oct;22(10):1350-60.
(204) Gomillion CT, Burg KJ. Stem cells and adipose tissue engineering. Biomaterials 2006 Dec;27(36):6052-63.
(205) Beauchamp JR, Heslop L, Yu DS, Tajbakhsh S, Kelly RG, Wernig A, et al. Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. J Cell Biol 2000 Dec 11;151(6):1221-34.
(206) Kallestad KM, McLoon LK. Defining the heterogeneity of skeletal muscle-derived side and main population cells isolated immediately ex vivo. J Cell Physiol 2010 Mar;222(3):676-84.
(207) Minguell JJ, Erices A. Mesenchymal stem cells and the treatment of cardiac disease. Exp Biol Med (Maywood) 2006 Jan;231(1):39-49.
(208) Ballas CB, Zielske SP, Gerson SL. Adult bone marrow stem cells for cell and gene therapies: implications for greater use
1. J Cell Biochem Suppl 2002;38:20-8.
(209) McLoon LK, Wirtschafter J. Activated satellite cells in extraocular muscles of normal adult monkeys and humans

14. Invest Ophthalmol Vis Sci 2003 May;44(5):1927-32.
(210) Antunes-Foschini RS, Miyashita D, Bicas HE, McLoon LK. Activated satellite cells in medial rectus muscles of patients with strabismus
1. Invest Ophthalmol Vis Sci 2008 Jan;49(1):215-20.
(211) Hollemann D, Budka H, Loscher WN, Yanagida G, Fischer MB, Wanschitz JV. Endothelial and myogenic differentiation of hematopoietic progenitor cells in inflammatory myopathies
1. J Neuropathol Exp Neurol 2008 Jul;67(7):711-9.
(212) Kottlors M, Kirschner J. Elevated satellite cell number in Duchenne muscular dystrophy
1. Cell Tissue Res 2010 Jun;340(3):541-8.
(213) Zammit PS, Heslop L, Hudon V, Rosenblatt JD, Tajbakhsh S, Buckingham ME, et al. Kinetics of myoblast proliferation show that resident satellite cells are competent to fully regenerate skeletal muscle fibers. Exp Cell Res 2002 Nov 15;281(1):39-49.
(214) Wang JF, Forst J, Schroder S, Schroder JM. Correlation of muscle fiber type measurements with clinical and molecular genetic data in Duchenne muscular dystrophy
3. Neuromuscul Disord 1999 May;9(3):150-8.
(215) Behan WM, Cossar DW, Madden HA, McKay IC. Validation of a simple, rapid, and economical technique for distinguishing type 1 and 2 fibres in fixed and frozen skeletal muscle. J Clin Pathol 2002 May;55(5):375-80.
(216) Goetsch SC, Hawke TJ, Gallardo TD, Richardson JA, Garry DJ. Transcriptional profiling and regulation of the extracellular matrix during muscle regeneration. Physiol Genomics 2003 Aug 15;14(3):261-71.
(217) Lucia A, Rivero JL, Perez M, Serrano AL, Calbet JA, Santalla A, et al. Determinants of VO(2) kinetics at high power outputs during a ramp exercise protocol. Med Sci Sports Exerc 2002 Feb;34(2):326-31.
(218) Havenith MG, Visser R, Schrijvers-van Schendel JM, Bosman FT. Muscle fiber typing in routinely processed skeletal muscle with monoclonal antibodies. Histochemistry 1990;93(5):497-9.
(219) Jennische E. Ischaemia-induced injury in glycogen-depleted skeletal muscle. Selective vulnerability of FG-fibres. Acta Physiol Scand 1985 Dec;125(4):727-34.
(220) Yun Z, Lin Q, Giaccia AJ. Adaptive myogenesis under hypoxia. Mol Cell Biol 2005 Apr;25(8):3040-55.
(221) Fluck M, Hoppeler H. Molecular basis of skeletal muscle plasticity--from gene to form and function
37. Rev Physiol Biochem Pharmacol 2003;146:159-216.
(222) Hiatt WR, Regensteiner JG, Wolfel EE, Carry MR, Brass EP. Effect of exercise training on skeletal muscle histology and metabolism in peripheral arterial disease. J Appl Physiol 1996 Aug;81(2):780-8.
(223) Williamson DL, Gallagher PM, Carroll CC, Raue U, Trappe SW. Reduction in hybrid single muscle fiber proportions with resistance training in humans
2. J Appl Physiol 2001 Nov;91(5):1955-61.
(224) Te KG, Reggiani C. Skeletal muscle fibre type specification during embryonic development. J Muscle Res Cell Motil 2002;23(1):65-9.
(225) D'Antona G, Pellegrino MA, Adami R, Rossi R, Carlizzi CN, Canepari M, et al. The effect of ageing and immobilization on structure and function of human skeletal muscle fibres
13. J Physiol 2003 Oct 15;552(Pt 2):499-511.

(226) Andersen JL, Gruschy-Knudsen T, Sandri C, Larsson L, Schiaffino S. Bed rest increases the amount of mismatched fibers in human skeletal muscle. J Appl Physiol 1999 Feb;86(2):455-60.
(227) Grossman EJ, Roy RR, Talmadge RJ, Zhong H, Edgerton VR. Effects of inactivity on myosin heavy chain composition and size of rat soleus fibers
4. Muscle Nerve 1998 Mar;21(3):375-89.
(228) Canepari M, Pellegrino MA, D'Antona G, Bottinelli R. Single muscle fiber properties in aging and disuse. Scand J Med Sci Sports 2010 Feb;20(1):10-9.
(229) Dodd SL, Vrabas IS, Stetson DS. Effects of intermittent ischemia on contractile properties and myosin isoforms of skeletal muscle. Med Sci Sports Exerc 1998 Jun;30(6):850-5.
(230) Gosker HR, van MH, van Dijk PJ, Engelen MP, van der Vusse GJ, Wouters EF, et al. Skeletal muscle fibre-type shifting and metabolic profile in patients with chronic obstructive pulmonary disease. Eur Respir J 2002 Apr;19(4):617-25.
(231) Albani M, Megalopoulos A, Kiskinis D, Parashos SA, Grigoriadis N, Guiba-Tziampiri O. Morphological, histochemical, and interstitial pressure changes in the tibialis anterior muscle before and after aortofemoral bypass in patients with peripheral arterial occlusive disease
1. BMC Musculoskelet Disord 2002;3:8.
(232) McGuigan MR, Bronks R, Newton RU, Sharman MJ, Graham JC, Cody DV, et al. Muscle fiber characteristics in patients with peripheral arterial disease
1. Med Sci Sports Exerc 2001 Dec;33(12):2016-21.
(233) Sinha-Hikim I, Taylor WE, Gonzalez-Cadavid NF, Zheng W, Bhasin S. Androgen receptor in human skeletal muscle and cultured muscle satellite cells: up-regulation by androgen treatment. J Clin Endocrinol Metab 2004 Oct;89(10):5245-55.
(234) Andersen JL. Muscle fibre type adaptation in the elderly human muscle. Scand J Med Sci Sports 2003 Feb;13(1):40-7.
(235) Lee WS, Cheung WH, Qin L, Tang N, Leung KS. Age-associated decrease of type IIA/B human skeletal muscle fibers. Clin Orthop Relat Res 2006 Sep;450:231-7.
(236) Kuang S, Charge SB, Seale P, Huh M, Rudnicki MA. Distinct roles for Pax7 and Pax3 in adult regenerative myogenesis
1. J Cell Biol 2006 Jan 2;172(1):103-13.
(237) Zammit PS, Relaix F, Nagata Y, Ruiz AP, Collins CA, Partridge TA, et al. Pax7 and myogenic progression in skeletal muscle satellite cells. J Cell Sci 2006 May 1;119(Pt 9):1824-32.
(238) Olson EN. MyoD family: a paradigm for development?
14. Genes Dev 1990 Sep;4(9):1454-61.
(239) Schultz E, Chamberlain C, McCormick KM, Mozdziak PE. Satellite cells express distinct patterns of myogenic proteins in immature skeletal muscle
1. Dev Dyn 2006 Dec;235(12):3230-9.
(240) Collins CA, Olsen I, Zammit PS, Heslop L, Petrie A, Partridge TA, et al. Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. Cell 2005 Jul 29;122(2):289-301.
(241) Zammit PS, Golding JP, Nagata Y, Hudon V, Partridge TA, Beauchamp JR. Muscle satellite cells adopt divergent fates: a mechanism for self-renewal? J Cell Biol 2004 Aug 2;166(3):347-57.
(242) Yoshida N, Yoshida S, Koishi K, Masuda K, Nabeshima Y. Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates 'reserve cells'. J Cell Sci 1998 Mar;111 (Pt 6):769-79.

(243) Seale P, Sabourin LA, Girgis-Gabardo A, Mansouri A, Gruss P, Rudnicki MA. Pax7 is required for the specification of myogenic satellite cells
2. Cell 2000 Sep 15;102(6):777-86.
(244) Roth SM, Martel GF, Ivey FM, Lemmer JT, Metter EJ, Hurley BF, et al. Skeletal muscle satellite cell populations in healthy young and older men and women. Anat Rec 2000 Dec 1;260(4):351-8.
(245) Bamman MM, Clarke MS, Talmadge RJ, Feeback DL. Enhanced protein electrophoresis technique for separating human skeletal muscle myosin heavy chain isoforms. Electrophoresis 1999 Mar;20(3):466-8.
(246) Talmadge RJ, Roy RR. Electrophoretic separation of rat skeletal muscle myosin heavy-chain isoforms. J Appl Physiol 1993 Nov;75(5):2337-40.
(247) Lu D, Chen B, Liang Z, Deng W, Jiang Y, Li S, et al. Comparison of bone marrow mesenchymal stem cells with bone marrow-derived mononuclear cells for treatment of diabetic critical limb ischemia and foot ulcer: a double-blind, randomized, controlled trial. Diabetes Res Clin Pract 2011 Apr;92(1):26-36.
(248) Schaper W, Scholz D. Factors regulating arteriogenesis
5. Arterioscler Thromb Vasc Biol 2003 Jul 1;23(7):1143-51.
(249) Dowell JD, Rubart M, Pasumarthi KB, Soonpaa MH, Field LJ. Myocyte and myogenic stem cell transplantation in the heart. Cardiovasc Res 2003 May 1;58(2):336-50.
(250) Lasala GP, Silva JA, Gardner PA, Minguell JJ. Combination stem cell therapy for the treatment of severe limb ischemia: safety and efficacy analysis. Angiology 2010 Aug;61(6):551-6.
(251) Dib N, McCarthy P, Campbell A, Yeager M, Pagani FD, Wright S, et al. Feasibility and safety of autologous myoblast transplantation in patients with ischemic cardiomyopathy. Cell Transplant 2005;14(1):11-9.

Appendix A:

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Patient Information Sheet

A study of blood vessel growth in peripheral vascular disease

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like further information. Take time to decide whether or not you wish to take part. Consumers for Ethics in Research (CERES) publish a leaflet entitled Medical Research and You. This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy maybe obtained from CERES, P0 Box 1365, London N16 OBW.

WHAT IS THE PURPOSE OF THE STUDY?

Heart attacks and stroke are major cause of disability and death in the western world. They are usually caused by atherosclerosis, a disease of the blood vessel which causes the narrowing of blood vessel lumen and subsequently impairment of blood flow to the organs affected. This can also affect the leg, a condition known as peripheral vascular disease, in which the impaired blood flow result in calf pain on exertion, also know as intermittent claudication. This can progress to end stage of the disease, known as critical limb ischaemia, which has a high rate of leg amputation. This research aims to study the molecular changes which stimulate blood vessel formation that occur in the skeletal muscles of the legs by comparing the legs with impaired blood flow to the unaffected legs.

WHY HAVE I BEEN CHOSEN? You have been chosen because you are going for surgery involving your leg.

DO I HAVE TO TAKE PART? It is up to you to decide whether to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

WHAT WILL HAPPEN TO ME IF I TAKE PART

On admission for operation, details will be obtained from you about your medical history, medication history, and examination of your legs will be performed. After your agreeing to the consent to enter the study, a small sample will of your leg muscle measuring approximately 1x1x1cm will be obtained during the operation.

WHAT DO I HAVE TO DO?

You need to do nothing additional apart from preparing for your planned operation.

WHAT ARE THE SIDE EFFECTS OF TAKING PART?

There is no additional side effects apart from that from your operation itself which will have been explained by the doctors caring for you.

WHAT ARE THE POSSIBLE DISADVANTAGES AND RISKS IN TAKING PART?

There is no major risk involved apart from the risks from the operation you are going for.

WHAT ARE THE POSSIBLE BENEFITS OF TAKING PART?

The information we get from this study may help us in the future to assess and treat patients with the similar conditions like yourself in the future.

WHAT IF NEW INFORMATION BECOMES AVAILABLE?

Sometimes during the course of a research project, new information becomes available about the treatment that is being studied. It usually applies to ongoing research project which involves treatment for a period of time. This does not affect you because your participation ends after you have completed the operation.

WHAT IF SOMETHING GOES WRONG?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for legal action but you may have to pay for it. Regardless of this, if you wish to complain about any aspect of the way you have been approached, or treated during the course of the study, the normal National Health Service complain mechanisms may be available to you.

WILL MY TAKING PART IN THIS STUDY BE KEPT CONFIDENTIAL?

All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital / surgery will have your name and address removed so that you cannot be recognized from it**.**

WHAT HAPPENED TO THE RESULTS OF THE RESEARCH STUDY?

The results of the study will be presented at scientific meetings and published in medical journals. To get a copy of the results, you can contact the trial co-ordinators whose name and addresses are outlined below. You will be told which arm of the study they were in. However, you will not be identified in any report / publications**.**

WHO IS ORGANISING AND FUNDING THE RESEARCH?

We have applied to a research charity / university for some money to sponsor this study**.**

WHO HAS REVIEWED THIS STUDY?

The study will be reviewed by the Royal Free Hospital and Medical School Ethics Committee.

CONTACT FOR FURTHER INFORMATION

Mr TK Ho, Clinical Research Fellow In Surgery, Royal Free Campus and University College, London, Rowland Hill, London NW3 2PF. Telephone: 02077940500 Extension: 5414
E-mail: t.ho@medsch.ucl.ac.uk or c.hart@medsch.ucl.ac.uk

Thank you for taking part in this study. You will be given a copy of the information sheet and a signed consent form to keep.

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ROYAL FREE AND UNIVERSITY COLLEGE MEDICAL SCHOOL
UNIVERSITY COLLEGE LONDON

CONSENT FORM

Title of Project: Study of Blood Vessel Growth In Peripheral Vascular Disease
Name of Researcher: Mr TK Ho/ Mr CA Hart

	Please initial box
1. I confirm that I have read and understand the information sheet dated ..... (version .....) for the above study and have had the opportunity to ask questions.	☐
2. I confirm that I have had sufficient time to consider whether or not I want to be included with the study.	☐
3. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	☐
4. I understand that sections of any of my medical notes may be looked at by responsible individuals from NHS or from regulatory authorities where it is relevant to my taking part in research. I also understand that samples of my muscles may be kept up to 6 months before analysed.	☐
5. I agree to take part in the above study.	☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature

Researchers: Mr TK Ho/ Mr CA Hart, Dept of Surgery, Royal Free Hospital, Pond Street, London NW3 2QG

Royal Free & Medical School Local Research Ethics Committee

Please reply to:

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23 August 2007

Mr Daryll M Baker,
University Department of Vascular Surgery
Royal Free Hampstead NHS Trust
Pond Street
Hampstead
London
NW3 2QG

Dear Mr Baker,

REC Ref: 29-2000 **The effect of peripheral vascular disease and its surgical reperfusion on microvascular tone mediators in man.**

Thank you for your letter dated 01.08.2007 reporting on the progressr of this study.

Your request to continue with the trial has been approved by Chairman's Action

Therefore, you are free to continue with your project.

Yours sincerely

Rosemary Brown
Ethics Committee Adviser

Appendix B:

BF35, Neonatal and Embryonic Myosin Were not Recognisable In Formalin Fixed Paraffin Embedded Adult Human Skeletal Muscle

The BF-35, neonatal and embryonic antibodies did not stain paraffin embedded human adult skeletal tissue after any applied pre treatment, at any concentration nor with any of the specified incubation conditions as listed below.

	BF-35, Neonatal and Embryonic Myosin					
	Pre-Treatment Used:					
	None	**Protein Kinase**	**EDTA 20m**	**Citrate 10m**	**EDTA PK**	**Citrate PK**
Concentration						
of Antibody						
[1:1]	0	0	0	0	0	0
[1:10]	0	0	0	0	0	0
[1:100]	0	0	0	0	0	0
	Performed at Room Temperature for I hour and 4^{o}C Overnight					

Optimisation of BF-35(all MHCs but IIx):, neonatal and embryonic myosin in paraffin embedded tissue with attempts to change pre-treatment, concentration of antibody and variation in incubation times. No staining was achieved under any conditions (0 = no stain).

Appendix B: Satellite Cell Markers Identified by Immunofluorescence

The process of dewaxing and rehydration of the samples, the blocking of endogenous peroxidases and microwave EDTA pH=9 pre treatment was followed as previously described (184)

Blocking of non-specific antigens was performed with 10% NGS for 30 minutes at room temperature before washing well with PBS three times for ten minutes. The primary antibody MyoD was incubated for an hour at a concentration of 1:50 before the slides were washed well again using PBS. All remaining steps were performed in the dark to avoid loss of the fluorescent signal. The secondary antibody, a red Fit C Goat anti Mouse (Vector Laboratories) was applied at a concentration of 1:200 for 30 minutes. A further wash was repeated before application of DAPi counterstaining for 10 minutes to mark nuclei (blue). PBS was used to wash the slides again and an aqueous mounting solution was used before application of the coverslip.

Appendix B: Satellite Cell Immunofluorescence of Satellite Cells

Although an immunofluorescent technique was attempted to investigate the number of active satellite cells present in the muscle sections by the use of MyoD as a marker. Although successful in marking satellite cells, this methodology was abandoned due to the brevity of signal life. There was simply not enough time to perform an adequate morphometric analysis of sections prepared in this way before signal was lost and the repetition of results was not helpful.

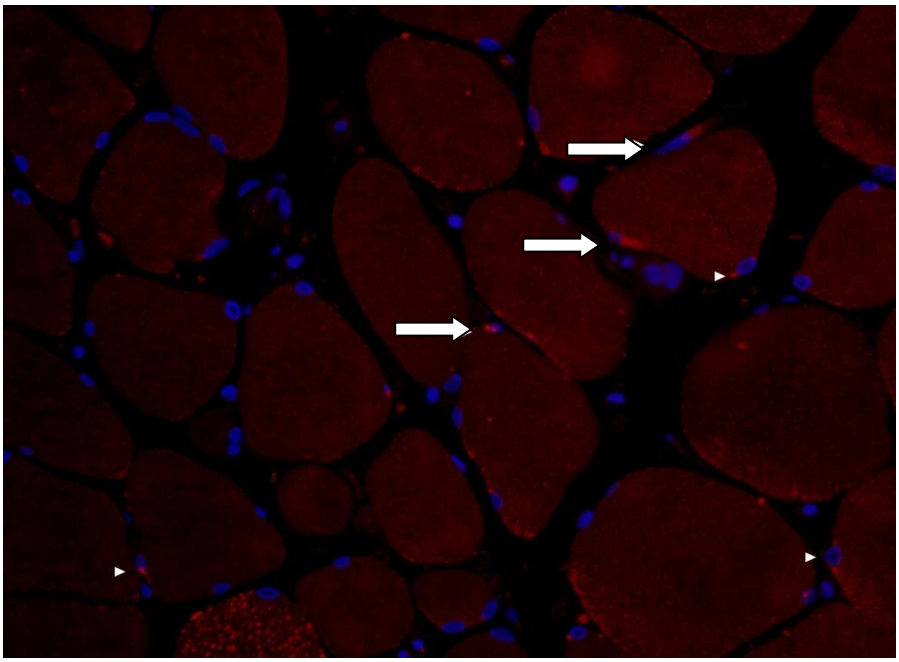

Figure 32: Immunofluorescent labelling of MyoD marking active satellite cells in ischaemic tissue. All nuclei emit a blue stain and MyoD positive nuclei are associated with a red stain, as indicated by white arrow heads (x200 magnification).

Appendix B: Alkaline Phosphatase Co-localisation Experiments

To demonstrate pax7 positive satellite cells under the basal lamina using a laminin antibody or CD34 to label satellite cells on the same slides to determine the number of quiescent cells, colocalisation via alkaline phosphatase immunohistochemistry was attempted.

Sections were rehydrated through xylene and ethanol over 10 minutes. Endogenous peroxidases were blocked in a bath of methanol with 3% hydrogen peroxide and sections were immersed in Tris/EDTA pH=9 and heat treated in the microwave for 20 minutes (184). 2.5 % Normal Horse Serum was added to the PAP encircled sections and flicked off. The primary antibody CD34 raised in mice was incubated on the tissue at a concentration of 1:50 for an hour at room temperature as previously described. After a good wash with PBS, the secondary antibody (horse anti-mouse alkaline phosphatase conjugated, Vector Laboratories, UK) was applied at [1:50] for an hour. After a further PBS wash as described, 10μls of A, B and C (Vector Laboratories, UK) was added to 1ml of 0.1mM HCl/TRIS (pH=8.2) 1:100. Vector red substrate was added for 30 minutes.

The slides were further washed in PBS and the slides were then blocked with 10% Normal Goat Serum for 30 minutes. Pax7 was incubated on the sections at [1:200] overnight at $4^{o}C$ and then washed in PBS. A Goat anti-rabbit secondary antibody (Vector Laboratories) at 1:200 was applied for 30 minutes and the remaining steps using the ABC method described earlier was performed.

Appendix B: ALP Colocalisation

This was attempted to colocalise CD34 and pax7 to examine the number of quiescent satellite cells present in each cohort of tissue.

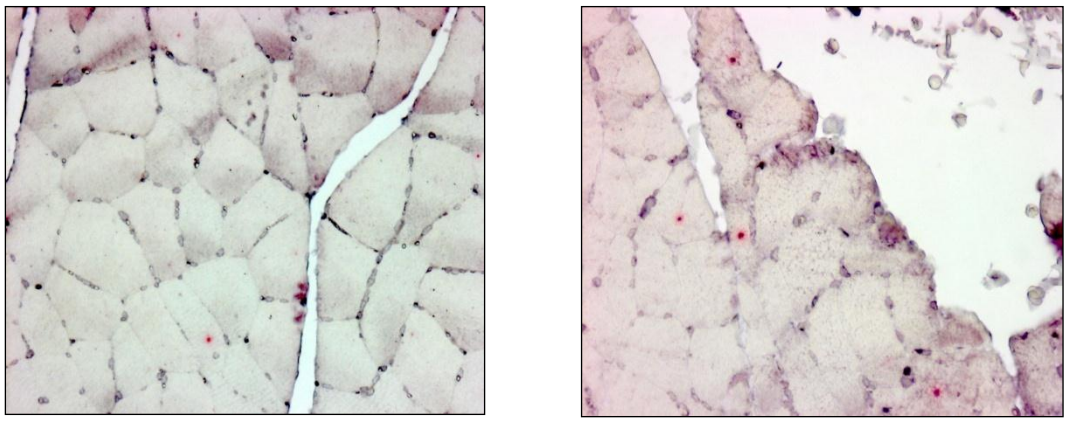

Figure 35a: CD34/pax7 Co expression in Control Tissue; 35b: CD34/pax7 in Ischaemic Tissue:

Background staining made this co-localisation technique too non specific and unreliable. The thin sections were also prone to damage through the vigorous pretreatment epitope retrieval and then long incubation periods made meaningful analysis impossible.

Appendix C:

Number of Fibres/ Field

CABG		Ischaemia	
A	28.8	C	69.4
B	32	D	84.2
F	25.8	E	75
I	27	G	37.8
J	23.5	H	49.4
L	24.6	K	18.2
N	22.6	M	57.6
O	16.2	R	32.4
P	35.2	S	35
T	21.6	U	45

Proportion of Fibres

Control	I	II	IIc	IIx	0
A	73	27	0	0	0
B	48	15	10	23	4
F	51	24	15	10	0
I	55	18	3	18	6
J	17	51	4	12	16
L	61	21	1	6	11
N	42	47	1	9	1
O	51	24	0	15	10
P	50	30	5	9	6
T	40	27	25	7	0

CLI

	I	II	IIc	IIx	0
C	76	7	14	1	2
D	57	39	4	0	0
E	58	12	16	14	0
G	60	9	25	5	1
H	93	0	7	0	0
K	49	41	10	0	0
M	80	16	0	0	4
R	75	13	8	3	1
S	42	31	22	1	4
U	68	20	12	0	0

Average CSA (All Fibres)

Control		CLI	
A	3643.08	C	3901.1
B	4215.51	D	2364
F	4687.53	E	1628.64
I	3929.25	G	3071.74
J	6136.58	H	3418.47
L	3782.83	K	5621.05
N	5532.44	M	1661.21
O	6709.32	R	3171.94
P	3732.51	S	3508.46
T	5201.86	U	2719.81

Average CSA by Fibre Type

Control	I	II	IIc	IIx	0
A	3989.29	2707	0	0	0
B	3827.12	5291.29	3530.76	4674.13	3916.92
F	4405.33	4618.51	3186.03	8924.83	0
I	3532.73	4070.82	2962.77	4336.74	6400.12
J	4837.17	6488.62	5761.12	6848.32	5955.13
L	3942.76	3470.86	5969.37	3419.09	3442.68
N	7118.53	4480.17	4504.88	3933.48	3791.73
O	6239.94	6890.25	0	8134.81	6530.7
P	3687.76	3354.67	1489.33	6531.45	3665.58
T	6087.11	4689.84	3367.67	8779.79	0

Ischaemic	I	II	IIc	IIx	0
C	4054.3	3598.36	3274.72	3664.52	3642.37
D	3189.82	1271.49	1248.09	0	0
E	1813.54	1421.89	1383.6	1319.85	0
G	3322.62	3889.09	2502.95	1806.89	1207.19
H	3543.68	0	1754.94	0	0
K	5976.24	5548.56	4177.84	0	0
M	1715.32	1339.37	0	0	1866.53
R	3219.86	3070.38	2461.87	4036.85	3983.66
S	4324.96	2756.34	3092.76	4028.54	2920.39
U	3013.35	2145.25	2014	0	0

Myonuclei/ 100 Fibres

Control		CLI	
A	3.77	C	3.24
B	7.8	D	6.97
F	4.73	E	4.12
I	3.05	G	9.73
J	5.64	H	4
L	3.42	K	4.68
N	3.51	M	7
O	4.87	R	4.4
P	4.6	S	4.39
T	3.64	U	4.11

Pathognomonic Changes

Control

	Angular Fibres	Central Nuclei	Clumping	Polygon Loss	Connective Tissue	Giant Cells	
A	0	0	Y	N	0	0	
B	1	9	N	N	0	0	
F	2	11	N	N	0	1 type I 1 type II	
I	0	5	N	N	0	0	
J	0	16	N	N	0	1 type I	
L	0	1	N	N	0	0	
N	1	2	N	N	0	0	
O	0	14	N	N	0	0	
P	0	6	N	N	0	0	
T	2	13	N	N	0	6 type I	N

CLI

	Angular Fibres	Central Nuclei	Clumping	Polygon Loss	Connective Tissue	Giant Cells	Z line loss
C	4	7	Y	Y	1	4 type I	Y
D	1	5	Y	Y	0	0	N
E	2	6	N	N	0	0	N
G	3	15	Y	N	1	0	Y
H	11	18	Y	Y	1	0	Y
K	11	3	Y	Y	1	4 type I 3 type II	Y
M	7	13	Y	Y	1	0	Y
R	7	20	Y	Y	1	0	N
S	2	3	N	N	0	0	N
U	12	3	N	Y	0	0	N

CD31 Endothelial Cell Marker

Control		**CLI**	
A	6.2	C	62.8
B	13.2	D	43.6
F	26	E	101.2
I	6.6	G	24.4
J	28	H	78.4
L	1	K	45.2
N	24.2	M	63.2
O	9.4	R	69.8
P	6.6	S	27
T	56.4	U	78.2

CD34 Haemopoietic Stem Cell Marker

Control		**CLI**	
A	46.6	C	69
B	41.8	D	112.8
F	22.2	E	105.8
I	36.8	G	49.8
J	34.6	H	86.4
L	9.2	K	54.8
N	29.8	M	52.8
O	36.4	R	78.2
P	49	S	70.4
T	40	U	90

pax7 Satellite Cell Marker (Quiescent AND Activated)

Control		**CLI**	
A	25.4	C	50
B	13.2	D	34
F	24.4	E	48.2
I	27	G	74.4
J	20.2	H	61
L	17.4	K	56.6
N	27.2	M	41
O	17.6	R	48.2
P	23	S	41.8
T	22.4	U	58.4

MyoD Myogenic Differentiation Marker

CABG		**Ischaemia**	
A	28.8	C	16.2
B	17.6	D	8.4
F	41	E	18.4
I	21.2	G	7
J	40.2	H	27.4
L	13.3	K	22.2
N	27	M	1.4
O	20	R	32
P	35	S	9.2
T	36.6	U	18.2

CD34/ pax7 Quiescent SC

CABG		**Ischaemia**	
A	6	C	17.4
B	6.8	D	16.8
F	6.2	E	20.2
I	6.8	G	17
J	3.2	H	19.4
L	3.6	K	21
N	10	M	15.2
O	4.8	R	19
P	6.8	S	18.6
T	8.8	U	20

MyoD/pax7 Activated/Proliferating SCs

CABG		**Ischaemia**	
A	9	C	3.4
B	6	D	2.4
F	14.6	E	3.8
I	3	G	3
J	8.6	H	6.2
L	4	K	4
N	8.6	M	0.4
O	3.4	R	6.4
P	6	S	1.4
T	6.6	U	2.6

Sample	No fibres	Nu/F	Slow	Fast	sc71	Hybrid IIc	CD31	CD34	pax7	MyoD	pax7 CD34	pax7 MyoD
A1	33	86/33	21	12	11	0	5	43	10	21	2	3
A2	32	94/32	29	7	9	2	4	58	34	37	5	14
A3	22	79/22	17	7	11	2	11	45	24	24	7	7
A4	23	107/23	18	5	9	0	7	38	36	34	7	11
A5	34	106/34	27	7	8	0	4	49	23	28	9	10
												9
B1	30	115/30	22	8	9	0	13	27	8	6	4	0
B2	29	91/29	9	20	22	0	16	31	10	18	7	5
B3	29	106/29	24	5	10	4	16	43	13	27	4	10
B4	35	113/35	24	11	11	3	3	53	23	17	9	11
B5	37	106/37	17	9	12	0	18	55	12	20	10	4
												6
C1	47	111/47	32	15	13	9	34	75	24	18	8	5
C2	38	141/38	21	17	16	7	45	50	63	8	22	2
C3	54	138/54	36	18	17	3	56	68	46	14	21	3
C4	59	147/59	41	18	18	8	58	79	65	19	20	4
C5	49	102/49	30	19	19	12	64	73	52	22	16	3
												3.4
D1	93	251/93	57	36	36	0	64	86	37	2	16	0
D2	52	183/52	38	14	14	0	24	112	24	9	16	2
D3	92	213/92	56	46	47	11	44	94	26	14	10	4
D4	86	209/83	53	56	56	17	33	133	28	12	19	4
D5	98	263/98	30	74	74	6	53	139	55	5	23	2
												2.4
E1	79	117/79	46	35	35	2	83	101	33	12	16	1
E2	89	234/82	57	47	20	0	115	148	63	16	34	4
E3	74	238/74	59	33	36	6	116	90	53	14	14	3
E4	59	207/59	41	33	50	5	85	66	50	28	16	6
E5	74	214/74	53	33	51	4	107	124	42	22	21	5
	375											3.8
F1	26	102/26	8	20	21	3	32	26	33	46	4	18
F2	21	104/21	8	10	12	0	20	18	14	48	4	16
F3	22	96/22	8	18	21	6	21	22	13	42	3	12
F4	24	112/24	10	14	15	1	36	26	29	26	12	15
F5	36	124/36	16	18	18	5	21	19	33	43	8	12
												14.6
G1	45	234/45	32	17	16	2	52	39	62	12	16	4
G2	40	242/40	27	16	19	8	65	60	81	4	21	3
G3	44	239/44	32	15	14	9	48	52	86	5	15	3
G4	25	210/25	21	11	13	7	55	57	65	5	17	3
G5	35	270/35	26	23	21	12	46	41	78	9	16	2
												3
H1	84	249/84	84	25	25	25	77	94	65	34	20	8
H2	40	160/40	40	2	15	2	77	83	67	32	21	8
H3	34	193/34	34	3	5	3	76	64	56	24	18	4
H4	37	148/37	36	2	9	1	64	70	41	21	15	6
H5	52	179/52	52	13	11	8	98	121	76	26	23	5
												6.2

Sample	No fibres	Nu/F	Slow	Fast	sc71	Hybrid IIc	CD31	CD34	pax7	MyoD	pax7 CD34	pax7 MyoD
I1	21	70/21	7	14	8	0	17	27	27	27	4	5
I2	25	110/25	21	5	8	1	3	41	33	13	10	4
I3	27	118/27	19	11	7	2	5	47	18	23	6	1
I4	29	66/29	14	15	7	1	4	37	33	20	7	2
I5	33	103/33	16	9	9	0	4	32	24	23	7	3
												3
J1	11	70/11	3	10	10	2	15	32	7	33	2	3
J2	28	112/28	9	19	15	0	33	42	23	50	1	10
J3	18	94/18	7	11	8	1	35	30	25	36	4	8
J4	29	115/29	19	10	6	2	19	38	19	43	6	10
J5	25	103/25	16	8	6	0	26	31	27	39	3	12
												8.6
K1	19	98/19	19	5	5	5	49	57	48	21	22	7
K2	18	87/18	18	4	4	4	46	50	56	24	25	3
K3	16	96/16	16	6	4	6	50	61	63	30	21	4
K4	21	79/21	20	1	1	0	39	57	67	17	18	2
K5	17	83/17	16	1	0	0	43	49	49	19	19	4
L1	17	71/17	15	2	4	0	1	13	5	9	4	4
L2	24	105/24	15	9	11	0	0	7	34	11	1	4
L3	25	71/25	13	12	12	0	1	5	25	21	3	7
L4	28	118/28	20	8	11	0	0	13	9	13	6	3
L5	29	102/29	23	4	6	0	3	8	14	14	4	2
												4
M1	68	230/68	68	6	5	6	54	63	50	1	13	1
M2	59	184/59	57	6	7	1	48	71	47	0	20	0
M3	51	169/51	50	1	2	2	63	51	36	2	16	0
M4	52	162/52	47	7	9	2	46	36	26	0	12	1
M5	58	166/58	56	2	1	0	57	43	46	4	15	0
												0.4
N1	27	63/27	9	18	11	0	15	24	25	24	8	5
N2	14	81/14	9	6	4	1	19	32	24	36	8	15
N3	22	101/22	5	17	14	1	19	30	28	28	14	11
N4	26	84/26	8	18	14	0	23	31	31	14	11	3
N5	24	62/24	20	4	6	0	25	32	28	33	9	9
												8.6
O1	16	60/16	16	1	2	1	13	33	24	14	7	3
O2	19	63/19	16	3	3	0	19	48	16	24	6	2
O3	16	83/16	6	10	9	0	3	42	18	23	2	2
O4	14	77/14	7	7	5	0	9	37	17	26	5	7
O5	16	74/16	3	11	5	0	3	22	13	13	4	3
												4.8

Sample	No fibres	Nu/F	Slow	Fast	sc71	Hybrid IIc	CD31	CD34	pax7	MyoD	pax7 CD34	pax7 MyoD	
P1	40	84/40	19	21	20	1	12	7	40	25	51	9	8
P2	32	62/32	12	22	22	2	15	5	62	16	36	6	7
P3	38	123/38	22	17	17	1	18	6	57	28	18	6	5
P4	28	103/28	14	15	15	1	27	7	33	30	38	7	4
P5	38	119/18	18	22	22	2	21	8	53	16	32	6	6
												6.8	6
R1	38	200/38	31	7	6	0	25	95	104	55	42	21	9
R2	31	135/31	29	6	4	4	27	44	48	71	31	14	5
R3	36	171/36	35	3	4	2	24	90	91	32	32	24	7
R4	24	119/24	20	6	5	2	32	37	50	49	26	14	7
R5	33	164/33	30	6	7	3	23	83	98	34	29	22	4
												19	6.4
S1	29	87/29	18	22	22	11	15	23	64	36	12	13	1
S2	36	103/36	16	21	21	5	17	24	62	58	3	19	0
S3	27	91/27	21	12	13	6	6	33	53	38	6	21	2
S4	42	115/42	16	36	34	10	14	17	80	40	9	18	2
S5	41	137/41	19	29	28	7	19	38	93	37	16	22	2
												18.6	1.4
T1	16	123/16	11	10	10	7	24	40	39	25	41	5	7
T2	20	98/20	14	15	14	9	24	45	29	23	34	7	8
T3	25	113/25	18	19	17	12	21	34	43	20	39	7	6
T4	20	104/20	12	17	17	9	20	38	44	21	32	16	5
T5	27	86/27	17	17	16	7	18	36	45	23	37	9	7
												8.8	6.6
U1	43	103/36	22	20	18	6	18	85	82	83	17	18	1
U2	51	121/51	42	16	18	7	22	76	84	56	22	17	2
U3	38	145/38	28	12	10	2	21	78	93	49	19	26	2
U4	48	128/48	37	20	18	8	25	72	98	52	18	19	5
U5	52	117/52	32	34	31	14	19	55	93	52	15	20	3

Presentations Arising From This Work:

Oral Presentation: SARS, London, December 2009

Satellite cell numbers and activity are increased in critically ischaemic muscle

Hart CA, Shiwen X, Tsui J, Hamilton G, Abraham D, Baker DM

Poster Presentation: The Vascular Society of Great Britain and Ireland (VSGBI) Annual Meeting, Manchester 28th-30th November 2007

Morphometric analysis of ischaemia-induced changes in human skeletal muscle

Hart CA, Khan K, Tsui J, Aslam R, Abraham D, Baker DM, VSGBI AGM,

Oral Presentation: 16th European Chapter Congress of the International Union of Angiology, Glasgow, October 2005

Increased Angiogenesis and Vascular Endothelial Subtype Characterisation In Human Critical Limb Ischaemia

Ho TK, Hart CA, Abraham D, Black C, Baker DM

Addendum to MD Thesis

The Effect of Critical Limb Ischaemia on Adult Human Skeletal Muscle

There have been a few areas of weakness of this thesis which have not been addressed in the Limitations Chapter. Some subjects have been incompletely detailed or in some cases omitted in the bulk of the thesis. These are described below and any shortcomings of the written thesis raised in the final viva voce examination are recognised and addressed here:

Immunohistochemistry:

One of the most obvious omissions was the failure to discuss the confirmation of successful immunohistochemical methodology through the use of positive controls in the assessment of muscle slides. Whilst the inclusion of Chapter 3.4.4 discusses Negative Controls, there is no mention in the text of the Positive Controls. This was performed at an early stage and certainly should have been included in the bulk of the text. Positive controls were recommended by the manufacturers of the antibodies used for the most part. The pax7 antibody from Aviva Biosystems did not have a recommended positive control from its data sheet but having discussed this with the company they advised the use of either cell lysates obtained from the company or the use of human bladder tissue. The latter was used as this tissue was cheaper to access and easier to analyse.

Myosin staining and correct identification, including that of sc71, was confirmed via personal communications with two experts in the field. The first, Simon M. Hughes, Professor of

Developmental Cell Biology, Medical Research Council Career, Kings College London, who not only looked over some of my slides but also very kindly suggested the Fast A4.74 myosin antibody in the first place and Dr Vika Smerdu of the Institute of Anatomy, University of Ljubljana, Slovenia who sent me some extremely helpful advice regarding the labelling of myosin fibre types. Both of researchers looked over examples of immunohistochemical stains sent to them electronically. Although these expert opinions were sought, these should have been formalised in some way and there should have been a discussion between us regarding validation of the results commented upon in the thesis.

Positive control tissue to ensure correct staining of satellite cell markers was obtained and prepared according to the guidelines set out by the manufacturers of the individual antibodies and the tissue used to confirm these were laid out below:

Pax7	Aviva Biosystems	Human bladder tissue
CD34	Dako	Human tonsil tissue
CD31	Dako	Human tonsil tissue
MyoD	Dako	Human rhabdomyosarcoma

<u>Power:</u>

Whilst an attempt was made to ensure that there were adequate numbers of muscle biopsies obtained, there were only n=10 in each group. This was clearly not a large number and this point has been raised as an issue in the Limitations Chapter. The number of fields and

measurements taken was felt to be adequate due to similar sample sizes quoted in the literature. Certainly before embarking on the project there should have been a greater attempt at ensuring the study was adequately powered and that samples were analysed in sufficient numbers. Although some papers that have been quoted have described methods of collecting morphometric data or quantifying nuclei counts via immunohistochemistry, many do not appear to be powered. This may be due to the fact that these are observational rather than interventional studies, or it may be a simple omission.

Whilst some papers advocate the optimum number of biopsy fields, or fibre characteristics to be measured [Garton, Smerdu, Behan] for the analysis of muscle in the clinical setting others are vague regarding the validation of their results. The use of the Mann Whitney U Test to compare two sets of continuous independent data in this work was correct and reduced the effect of any outliers due to the analysis of median rather than mean, other groups have used χ^2 and degrees of freedom to analyse and even validate results. Validation is a separate issue and is discussed below.

Validation of results:

The above was not well described in the bulk of the thesis and certainly should have been addressed more clearly. In this study, the morphometric analysis of ten fibres of each sample was undertaken at a later date and compared to the original data set. However, the Mann Whitney U Test was utilised for this task and there was no significant difference was found between the original data set and that collected afterwards for validation purposes but it must

be acknowledged here that this was not the correct test for validation. The Analysis Of Variance (ANOVA) test or an F-Test would have been the correct test to validate my results.

One of the weak points of this thesis was the lack of advice from a medical statistician. If such had been employed from the beginning of the project, this would have thus prevented criticism of the collected data, which would have stood up to greater scrutiny and would have been much more credible, with the correct validation performed of the collected data.

Western Blots:

These were found to be extremely difficult and the experimental technique took some time to optimise. In fact, a satisfactory conclusion to the question of whether satellite cell protein expression could be quantified was achieved by visiting an experienced research group based at Leicester University. They made the following modifications to the methodology I had employed (acknowledgements to Dr Nisha Patel), which involved the use of TBS rather than PBS and the use of bovine serum albumin as well as evaporated milk to block the membrane. This group also recommended the use of commercially sourced 4%:20% gels rather than those made up locally.

1. Block the membrane in 5% BSA for 1 hour.

2. Incubate the membrane in 1 in 2000 dilution of primary antibodies overnight - e.g 2.5 microlitres in 5mls of 1XTBS.

3. Wash the membrane 3x for 10 minutes in 1x TBS.

4. Incubate the membrane in secondary antibody (mouse 1 in 2000 dilution) again for 1 hour.

5. Wash the membrane 3x for 10 minutes in 1 x TBS.

6. Develop the membrane.

The Western blots were therefore prepared in this optimised way and yielded a much better result with much cleaner signalling.