UNIVERSITY OF BIRMINGHAM

CREATINE TRANSPORTER CONTENT AND LOCALISATION IN HUMAN SKELETAL MUSCLE: THE EFFECT OF AGEING AND DISUSE

by

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GENERAL ABSTRACT

The maintenance of intramuscular creatine (Cr) is important for sustainability of skeletal muscle mass across the life-course. Creatine transporter (CreaT) is the only known specific Cr transporter which embedded in the cell membrane as a gate-keeper that transports Cr from blood into skeletal muscle. The impairments of structure and functions in CreaT will lead to insufficient intracellular Cr concentration, resulting in the disturbance of cellular energy homeostasis, metabolic disorders, muscle atrophy and neurological disorders. Impaired energy homeostasis could also have implications for dysregulated proteostasis, and muscle atrophy. Maintenance of proteostasis is essential to sustain skeletal muscle mass and therefore vital to health across the life-course. This is especially important to populations in the context of musculoskeletal ageing and scenarios of disease/injury. In this regard, detections of tissue CreaT content may aid the clinical diagnosis of diseases. As such, to understand the CreaT regulation mechanisms and its role in Cr metabolism can help the development of therapeutic interventions to improve intramuscular Cr levels and support muscle mass in health and disease. However, the role of CreaT in modulating Cr metabolism in skeletal muscle remains largely unexplored. The existing measurement techniques lack precision to fully explain the regulatory role of CreaT in different physiological scenarios of muscle deterioration. Accordingly, **Chapter 2** of this thesis validated and optimized an immunofluorescence microscopy technique and provided a more precise approach to identify CreaT protein expression and distribution in human skeletal muscle. A new method of calculation was designed and developed to identify CreaT protein intensity in specific sub-cellular regions. By using these developed methods, **Chapter 3** of this thesis investigated whether CreaT distribution and sub-cellular expression is altered in older age. A sub-set of quadriceps muscle biopsy samples from 6 young and 6 older

healthy individuals were involved in study to identify CreaT content and distribution, Creatine kinase (CK) and high-energy phosphagens. No significantly difference of CreaT content was observed between young and older individuals but with a declined concentration of phosphocreatine (PCr) ($P<0.05$) in older muscles. CreaT protein localisation was determined to predominantly distribute at the cell peripheral in both young and old individuals ($P<0.05$) but with fibre type specific difference in old individuals. These results demonstrated that Cr metabolism did alter by ageing, however, CreaT content remains relatively unaffected. **Chapter 4** of this thesis investigated the effect of immobilization on CreaT protein expression and distribution in human skeletal muscle. The 7-days leg immobilization trial was undertaken in which 15 healthy young individuals. The results elucidated that 7 days immobilization lead to a declined muscle isometric strength and fat free mass ($P<0.01$), in addition with a reduced type II muscle fibre cross-section area (CSA) ($P<0.05$). CreaT protein content was greater in immobilised leg compared control leg ($P<0.01$). CreaT was mainly distribute at the cell peripheral in both immobilised and control legs with fibre type specific difference ($P<0.01$). These outcomes determined that the greater muscle fibre CreaT expression with immobilization may have important implications for skeletal muscle energy production during disuse-induced atrophy and in the recovery of muscle mass upon re-loading.

In conclusion, this thesis provides a novel immunofluorescence approach to investigate CreaT activities in human skeletal muscle. This developed method provides valuable insight into the role of CreaT in alteration in intramuscular Cr metabolism across the spectrum of health and disease. In addition, this thesis enhances our understanding of CreaT protein expression and sub-cellular localisation in the role of Cr in skeletal muscle physiology.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my gratitude to Dr Leigh Breen for giving me the opportunity to become a PhD. Leigh, words are powerless to express my gratitude. I really sincerely thank you for all the concern, help and supports you provided to me. Thank you always being patient to talk with me at those most important moments in my life, giving me directions when I was in confusion and shaping my science career. Your desire for knowledge and passion for science is inspiring me and improved my enthusiasm for research. I thank you for the doors you always opened for me. Hope we will have many opportunities to work together in the future. Additionally, I would like to thank Professor Craig Sale of University of Nottingham Trent University, Dr Sarah Aldred and Dr Gareth Wallis of University of Birmingham for taking time out of their busy schedules to serve on my PhD defence committee.

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Last, but certainly not least, my heartily thanks go to the love of my life, Shuai, thank you for your company, meticulous care and endless love during my Ph.D. Thank you for always tolerating my temper and depression, giving me encouragement and confidence throughout difficult times.

To my wonderful family I dedicate this thesis. I thank you for motivating me during the difficult times and all the financial supports. Thank you for your understanding and all the loves you gave to me. Thanks dad, the words give to me 'Perseverance is a virtue' is sticky in my life. They are the most important things being supported and encouraged me through my PhD life.

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CONTENTS LISTING

LIST OF CONFERENCE COMMUNICATIONS AND PUBLICATIONS

Data from the postgraduate study resulted in the following conference communication:

Novel Insights into Human Skeletal Muscle Creatine Transporter Expression as a Function of Age. European Sports Science Conference (ECSS), Prague, July 2019 – Oral communication.

Mechanisms of intramuscular creatine metabolism and transport in human skeletal muscle: Effects of ageing in/activity and supplementation. Postgraduate Research meeting, School of Sport, Exercise and Rehabilitation Science, University of Birmingham, Birmingham, December 2017 – Oral communication.

LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AGAT	L- Arginine: glycine amidinotransferase
Akt	Protein kinase B (PKB)
ALM	Appendicular lean mass
AMP	Adenosine Monophosphate
AMPK	5' Adenosine Monophosphate-activated protein kinase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AU/a.u.	Arbitrary unit
BBB	Blood-brain barrier
BMI	Body mass index
BSA	Bovine serum albumin solution
CK	Creatine kinase
Cl	Chloride
CoA	Coenzyme A
Cr	Creatine
CreaT	Creatine transporter
CTD	Creatine transporter deficiency
DNA	deoxyribonucleic acid
DEXA	Dual energy x-ray
EDTA	Ethylenediaminetetraacetic
EGTA	Ethylene glycol tetraacetic acid

eNOS	Endothelial nitric oxide synthase
eNOS ser^{1177}	eNOS phosphorylated at $serine^{1177}$
FFM	Fat free mass
FM	Fat mass
GAA	Guanidinoacetate
GAMT	Guanidinoacetate N-methyltransferase
GLUT4	Glucose transporter type 4
IGF	Insulin-like growth factor
IGF-1	Insulin-like growth factor-1
MHCI	Myosin heavy chain I
MHCII	Myosin heavy chain II
Na	Sodium
NGS	Normal goat serum
NO	Nitric oxide
NPB	Net protein balance
PCr	Phosphocreatine
PBS	Phosphate buffered saline
SC	Satellite cells
SLC6	Solute carrier family 6
SLC6A8	Solute carrier family 6 member 8
TBST	Tris-buffer saline with 0.1% Tween
TCr	Total creatine
TM-BBB	Brain capillary endothelial cells
WGA	Wheat germ agglutinin

AUTHOR'S DECLARATION

I completed the development of the immunofluorescence microscope technique and optimized the method described into details in chapter 2 and applied this technique in all experimental chapters in this thesis. I was taught the quantification method by Dr Sophie Joanisse.

The studies detailed in chapter 3 were run in collaboration with Dr Benoit Smeuninx. Ben conducted all the preliminary testing and training experiment work and collected all muscle biopsy samples. I finished the sample sectioning, immunofluorescence histology staining, wester blots, imaging capture, quantification and statistical analysis.

The experimental study detailed in chapter 4 was run in collaboration with Dr Leigh Breen, Miss Sophie Edwards and Mr Yusuke Nishimura. I was present during testing sessions to assist with muscle biopsy collection and storage, blood sample handling, Sophie and Dr Leigh Breen organized and carried out the clinical experiments with the assistance of DL and other group members. I performed all the immunofluorescence histology staining, imaging capture, quantification and statistical analysis of the data.

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Chapter 1

General Introduction

1.1. The Importance of Skeletal Muscle in Humans

Skeletal muscle accounts for approximately 40%-50% of body weight and functions to maintain posture, aids locomotion, and represents an important nutrient store and metabolic regulator (45). The architecture of skeletal muscle is exquisitely arranged and muscle mass can be generally determined by number and size of muscle fibres. Each muscle fibre contains nuclei, mitochondria, T-tubules, satellite cells, sarcoplasmic reticulum, bundle of myofibrils and is surrounded by a sarcolemma (105). The sarcolemma is associated with multiple proteins and physically connects to the internal myofilament, which forms a protein complex. These protein complexes (principally actin and myosin) play important roles in muscle contraction and relaxation. Skeletal muscle contains 50-75% of whole body proteins and around 30-50% whole-body protein turnover (17, 32). Dysfunction of these proteins may result in muscle disorders that manifest in atrophy (62). Additionally, due to the large amount of skeletal muscle in proportion to body mass, it is an important site for whole-body metabolic regulation (75).

Theoretically, skeletal muscle mass is reliant on overall protein net balance. The net balance of muscle protein depends on the processes of protein synthesis and degradation, and both of the processes are associated with various factors such as nutrient ingestion, hormonal status, physical activity and disease (7, 136, 165). Muscle strength is important in supporting posture and movement, which strongly influence activities of daily living. It has been well established that muscle strength in adults reaches peak levels between 20 and 35 years of age (56) before gradually declining at an average rate of 6-8% per decade from the fourth or fifth decade of life onwards (33) (**Figure 1.1**). The reduction of skeletal muscle mass and strength are commonly accompanied with increasing risk of disability and mortality (1, 22). Therefore, maintenance of skeletal muscle mass is vital to maintain physical function, quality of life and health-span. In addition to the roles defined above, skeletal muscle contributes to amino acid storage and

energy production for muscle contraction. Muscle contraction and cellular activities are mostly reliant on the production of adenosine triphosphate (ATP) (136).

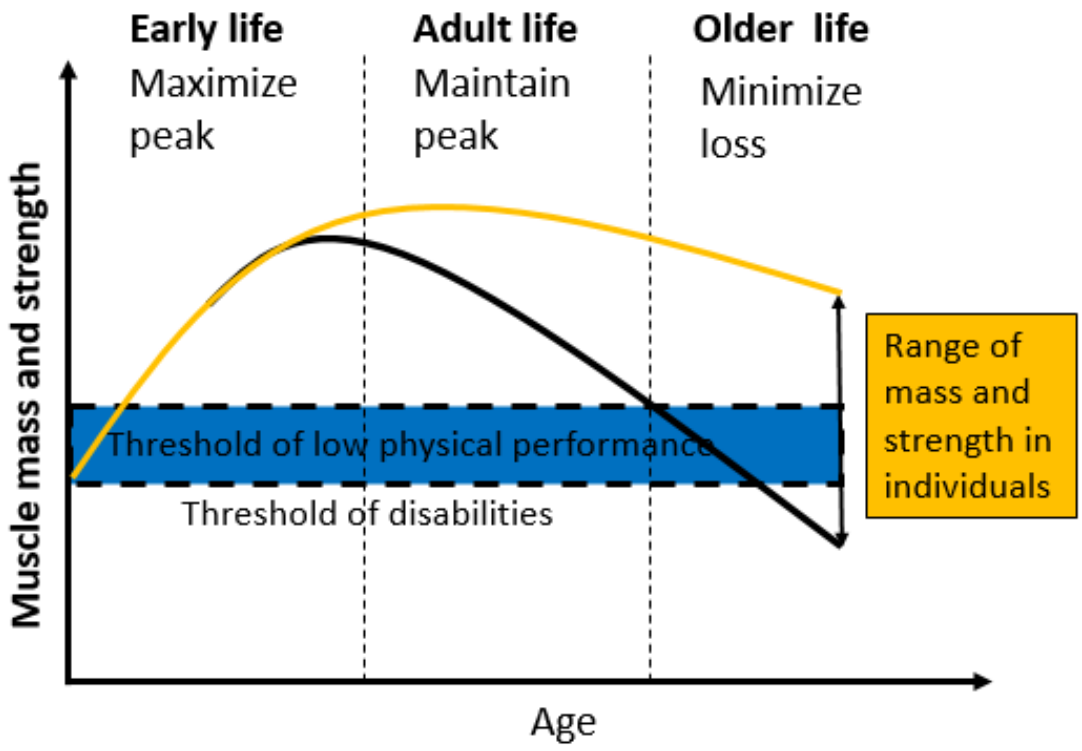

Figure 1.1 *Changes of muscle mass and strength in lifespan. Muscle strength in adults reach to the peak levels between 20 and 35 years of age before gradually declining at an average rate of 6-8% per decade start from the fourth to fifth decade until further* (33).

1.2. Human Skeletal Muscle Energy Production

1.2.1 ATP Generation

Muscle contraction requires energy, which is provided by ATP synthesis. There are three different metabolic reactions that produce ATP to maintain tissue homeostasis and contraction in human skeletal muscle (60, 167) (**Figure 1.2**). The first pathway to generate ATP is the phosphagen system also called ATP - PCr (Phosphocreatine) system or CK-PCr (Creatine

Kinase). This is the fastest pathway to generate ATP via PCr and does not demand the presence of oxygen (60, 178). The ATP - PCr system works as the predominate energy system, providing an immediate source of energy to support high intensity exercise lasting ~10 seconds (19). However, this process is highly reliant on the amount of PCr and ATP storage in skeletal muscle and may explain why fatigue occurs rapidly. The second reaction is glycolysis, which generates ATP from blood glucose or muscle glycogen and provides energy to meet the skeletal muscle requirements from 30 seconds up to 2 minutes of exercise. Glycolysis is thought of as a bridge-pathway to provide high ATP flux for a limited period between high and moderate exercise intensities (60, 133). The third pathway is mitochondrial respiration, which generate ATP from glucose and fat oxidation in mitochondria. During prolonged activity, this oxidative phosphorylated pathway is the major source of ATP, which is catalysed at the inner and outer mitochondria membranes via mitochondria creatine kinase (CK-Mt) (87, 178). These three reactions work individually or combined with each other to continually supply energy to muscle cells during exercise or maintain cell homeostasis at rest (**Figure 1.3**).

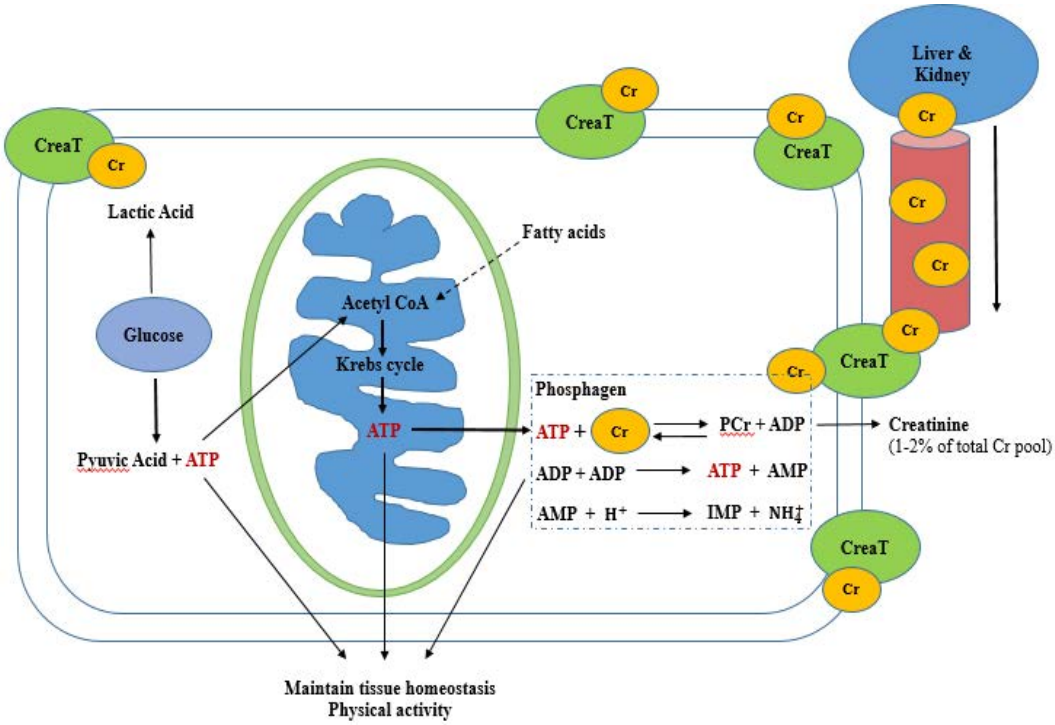

Figure 1.2 *Human skeletal muscle ATP generation systems. Three pathways involve in a skeletal muscle ATP generation. During short intensive exercise, skeletal muscle generates ATP mainly via the anaerobic pathways. Cr is transported into skeletal muscle cells vis its specific transporter protein CreaT and participate in the phosphagen pathway to generate 1 molecule of ATP by catalysing CK, when the storage of PCr exhausted, glucose becomes involved in the process of ATP production. Glucose participates in both anaerobic and aerobic processes to generate ATP. When the oxygen is insufficient, glucose breakdowns into pyruvate and lactate to generate 2 molecules of ATP. During prolonged exercise, glucose or glycogen and free fatty acids are the major sources of ATP generation with sufficient oxygen. In this oxidative ATP generation process, aerobic glycolysis occurs in the cytoplasm to produce ATP from glycogen where free fatty acids take place in mitochondrial and transform into Acetyl CoA to enter Krebs cycle to produce ATP 36 molecules of ATP.*

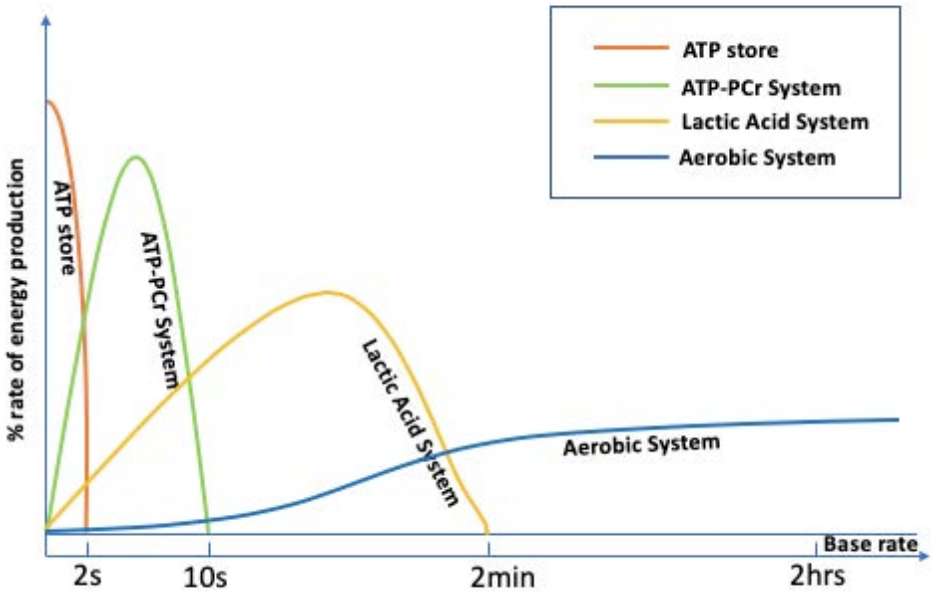

Figure 1.3 *Skeletal muscle energy continuum production during exercise and at rest. The storage of ATP is quickly depleted within 2s. The three energy systems are combined to work together to replenish ATP to support muscle contractions.*

1.2.2. The Phosphagen System

The phosphagen system uses phosphorylated creatine (PCr) to generate ATP at a very rapid rate. This system typically consists of three reactions. The first reaction is phosphocreatine together with Adenosine Diphosphate (ADP) and protons (H^+) to produce ATP and Cr via creatine kinase (CK) catalysing; the second reaction is ADP producing adenosine monophosphate (AMP) and ATP by catalysing adenylate kinase (AK); and the third reaction is the production of AMP via AMP deaminase and, eventually, the production of ammonia ($NH4^+$) (60). The phosphagen system serves three major functions to ensure the efficiency of energy utilization (167). Firstly, it functions to several physiology processes and plays essential roles in numerous metabolic activities. Secondly, this system functionally produces AMP which presents as an effective activator in the phosphorylase process to produce immediate fuel for glycolysis (87, 174). Thirdly, this system play roles on maintaining the AMP to ADP ratio in skeletal muscle required to sustain sufficient free energy release during ATP hydrolysis (87). This mechanism will be illustrated and described in further detail in section 1.4.2.

A number of activities are highly reliant on the phosphagen system due to its advantage in providing a large amount of ATP rapidly during high intensity exercises. Although the capacity of total PCr is limited, ~85% of PCr can be resynthesized within 4.5 min (60). The total Cr pool can be increased by using oral Cr supplementation or other combined supplementation strategies (37, 66, 72).

1.3. Skeletal Muscle Creatine Synthesis and Metabolism

1.3.1. Creatine (Cr) Homeostasis

Creatine is a non-essential dietary compound and can either be ingested from food or be produced endogenously (29, 85). Cr comes from the Greek word for 'flesh' and was first found in meat in 1835 (151, 181). Subsequent to this in 1927, PCr was been found to hydrolyse in skeletal muscle under contraction (181). Cr is mainly synthesized in kidney, liver and pancreas by using three different amino acids which include arginine, glycine and methionine, catalysed by two enzymes, L-arginine:glycine amidinotransferase (AGAT) and guanidinoacetate N-methyltransferase (GAMT) (14). Cr is principally transported to high energy demanded tissues via blood, such as skeletal muscle (5), and is degraded to creatinine via the kidneys. The daily excretion of creatinine is ~1-2% (~2g per day) (14, 144). Therefore, dietary Cr consumption is important to maintain the skeletal muscle Cr pool to support muscle contractile activities and a stable metabolic homeostasis. The total body Cr pool (Free Cr + PCr) is ~120 -130 mmol/kg dry mass in human (72, 147). However, the range could fall between 70 and 160 mmol/kg in different populations (13). In humans, nearly 95% of Cr, of which 60-70% is in the form of PCr stores (~70 mmol/kg dry muscle) (133) resides in skeletal muscle to maintain intracellular levels of ATP via PCr degradation and supply energy to all cells in body during exercise (147, 167). The major physiological function of Cr is to buffer the energy concentration in tissue to meet the high-energy requirement, especially in skeletal muscle and the brain. The full biosynthesis of Cr is shown in **Figure 1.4**.

The key function of intramuscular Cr is represented as a temporal energy shuttle (176) to restore ATP and maintain energetic homeostasis in human skeletal muscle (70). The importance of Cr is not only reflected as an energy buffer and energy carrier but also play roles in cellular energy

metabolism. In the past two decades, researchers have found that Cr plays a role in preventing excessive levels of ADP in cells and to control the ration of ADP to ATP. This is important for the production of oxidative energy in mitochondria. Moreover, Cr is also considered to be an antioxidant that decreases the damage of oxygen free radicals to cells. Cr aids to buffer cell PH. This is important especially during high intense exercise. Furthermore, Cr is responsible for transferring energy from the mitochondria to cytoplasm via variety of different forms of CK (108).

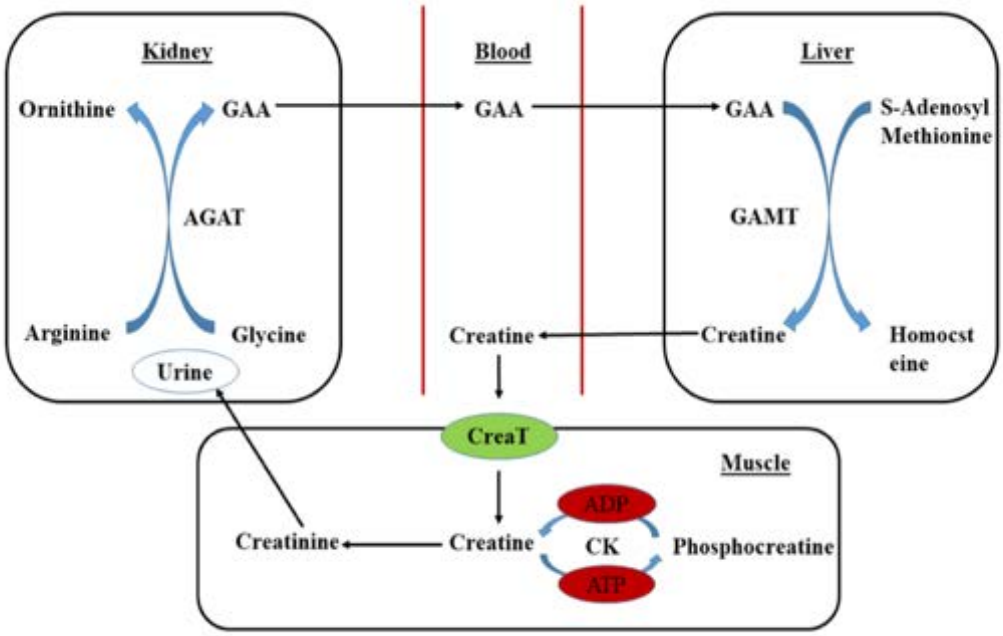

Figure 1.4 *Cr biosynthesis. Dietary and endogenous Cr transport into skeletal muscle via blood stream with a specific sodium/chloride depended creatine transporter protein. The endogenous Cr is firstly synthesised by Glycine and arginine which catalysed by AGAT and process GAA and Ornithine, GAA then transport to liver through blood. In liver, GAA and S-Adenosyl methionine catalysed by GAMT to form of Cr. Cr releases to blood stream from live and enters into skeletal muscle tissues via creatine transporter. About 1-2% of Cr degrade into creatinine and excrete from muscle cell via urine into kidney.*

1.3.2. Creatine Kinase (CK) Activity

The production of ATP via PCr/Cr is a rapid process, which requires enzymes to catalyse the reaction. CK is the enzyme which catalyse the reversible exchange of high energy phosphate between PCr and Cr to product ATP and ADP (23). There are at least four CK isoforms participant in metabolic activities inside body, which are mostly distributed in the skeletal muscle (CK-MM), in heart muscle (CK-MB), in brain tissue (CK-BB) or mitochondria (CK-Mt) (10, 174). Each CK isoenzyme has a particular function due to its specific localisation (174, 176), whereas CK-BB is correlated with several brain diseases (150) and CK-MB is related with some myocardial disorders. CK-MM is mainly bound to myofibrillar protein is the main source of total CK concentration in serum (23). CK-MM is closely coupled to glycolysis and generates ATP to maintain muscle tissue activities, impaired CK-MM activities are associated with muscle dysfunction (10). CK-Mt is closely coupled to the electron transport chain, uses mitochondrial ATP to resynthesize PCr and has been linked with mitochondrial myopathies (112).

Functionally, total CK levels are effected by several factors, such as gender (22), age (174), muscle mass and physical activity (10). Young healthy males have high serum CK levels, which slightly decrease with ageing (88) (**Figure 1.5)**. Healthy females present lower levels of CK than males at rest and post-exercise (10). High levels of serum CK after exercise training in healthy individuals may represent muscle tissue damage and fatigue (23). However, elevated CK levels present at rest may be a biomarker of clinical diseases (23, 155). Therefore, clinical measurement of CK activities and characterization of its isoforms have become key predictors of diagnosing myopathies, risk of cardiovascular diseases (26, 155), neurological disorders (89), osteoarthritic disorders (6) and obesity (71).

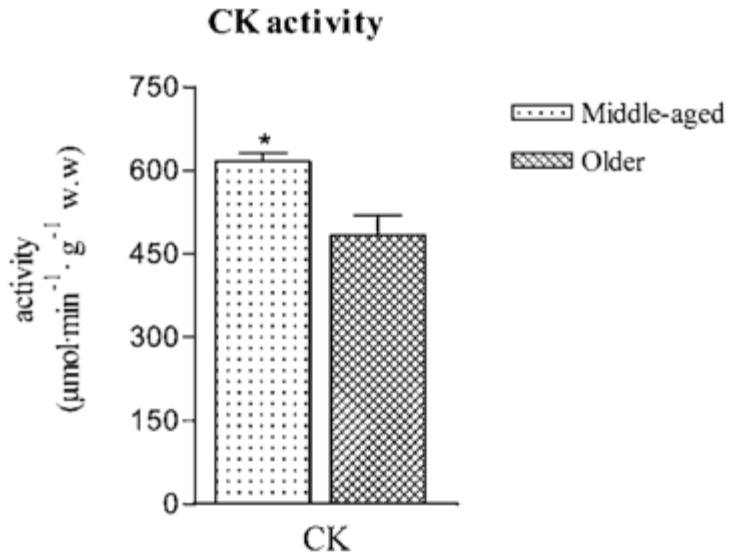

Figure 1.5 *Effect of age on skeletal muscle creatine kinase (CK) activity* (88).

1.3.3. Creatine Utilization During Muscle Contraction

Energy is important for cells to complete multiple biological activities and can be sourced from food. When food enters body, it can be digested into fat, protein and carbohydrate, and all of these need to be prior converted into ATP and then supply energy to cells (83). Therefore, ATP can be considered as a medium between food and cellular energy. Whilst cells require ATP to function, however, the amount of ATP in each cell is limited (15). Therefore, the maintenance of sufficient ATP is important to sustain cellular biological function during muscle contraction, especially high intensity exercise.

During moderate intensity exercise, absence of any regeneration, the total amount of intramuscular ATP is only sufficient to sustain muscle contraction for ~10 seconds (36). ATP is continuously re-generated to meet the energy requirements of working muscle. Although the main source of muscle ATP regeneration is derived from the breakdown of fat and glycogen, ATP regeneration via these pathways requires a longer time than PCr regeneration. Therefore,

in short duration high-intensity exercise, such as weightlifting or sprinting, the speed of ATP re-generation from fat and glycogen will not be enough to preserve the energy demand for continued muscle contraction. In these situations, the energy required is mainly supplied by high energy phosphagens. Muscle contractions can lead to an immediate increase in intramuscular ADP concentration, which results in hydrolysis of PCr by CK catalyzation. PCr is an important high energy phosphate that can be used to quickly replenish ATP. However, the amount of PCr is limited in skeletal muscle, and is typically depleted during high-intensity exercise. The reduction of PCr concentration can further result in decreased exercise performance leading to fatigue (20, 87). ATP can be rapidly re-generated from this reaction without any oxygen and preserve muscle contraction for approximately 10 seconds. Therefore, an elevation of intramuscular Cr levels can improve fatigue resistance and endurance capability during high-intensity exercise. Previous research showed that the Cr supplementation intake can increase the total Cr pool and, therefore, elevate cellular Cr/PCr concentration. This elevation can enhance the concentration of PCr and provide more energy to support exercise contraction during for high intensity, short duration and repeated bound of exercise (140). The potential roles of Cr and PCr in skeletal muscle metabolism and energy delivery are present in **Figure 1.6**

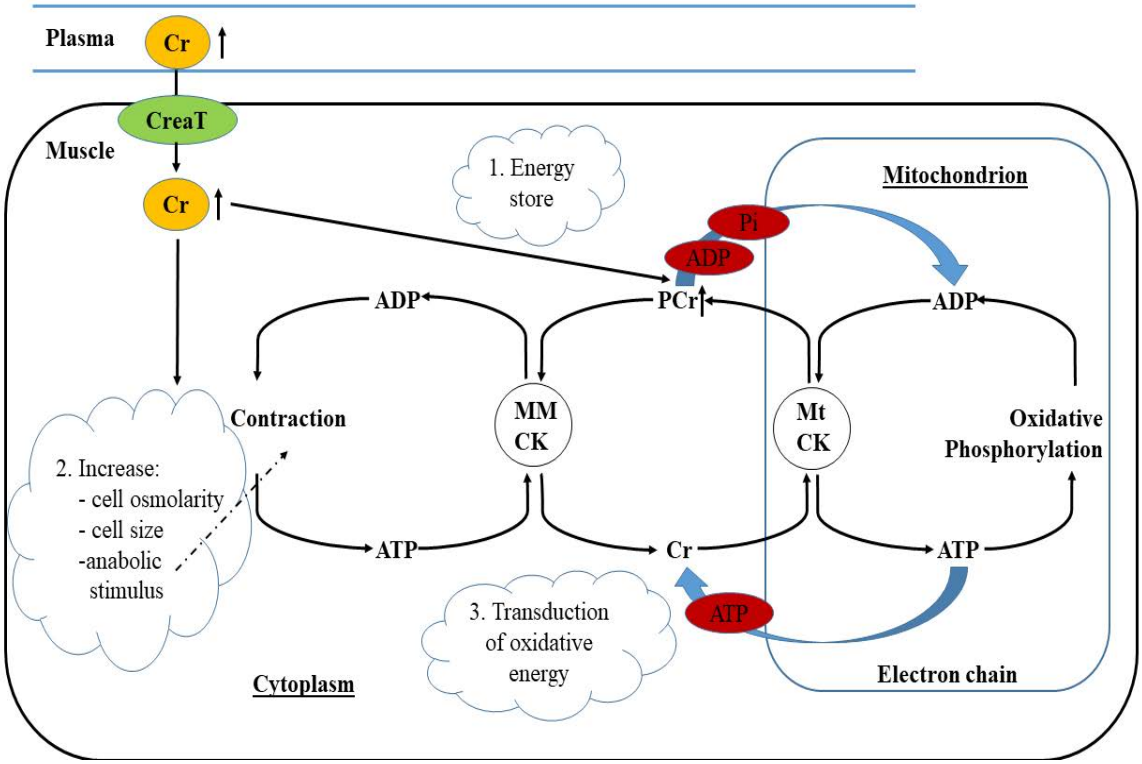

Figure 1.6 *Schematic representation of the potential roles of Cr and PCr in skeletal muscle metabolism and energy delivery* (149).

1.3.4 The Role of Creatine Supplementation

Creatine supplementation has been used as a diary supplement to enhance an athlete physical performance for over 150 years. Cr supplementation individually or combined with resistance training enhances skeletal muscle mass and strength gains (34, 157), improves muscle performance and fatigue resistance (21), enhances cognitive function (8, 124), can prevent osteoporosis (39, 69), increases muscle cell blood glucose utilization (80, 126) and increases neuroprotection (1, 92). These ergogenic and health benefits are mainly governed through the elevation of PCr/Cr storage and its re-synthesis (66, 163) to generate more ATP. This has previously been observed in a human study which showed the muscle total Cr pool can be enhanced by ~10%-20% following Cr supplementation (134). The enhancement of

intramuscular Cr pool may relate to the increased lean muscle mass. In most cases the lean muscle mass enhancement when resistance exercise is combined with Cr supplementation is within the range of 1–2 kg (54, 153). The mechanisms through which Cr supplementation modulates muscle Cr metabolism and function to improve of muscle mass and performance are yet to be clearly defined. Based on previous findings, this increased muscle mass/size is potentially due to stimulation of muscle protein synthesis (48), inhibition of muscle protein breakdown (32) or augmented water retention (86, 121). It has been reported that the increased muscle mass after Cr supplementation is related to the enhanced number of muscle satellite cells and cell nuclei (45, 114). Basically, the nucleus is a fundamental cellular component, which contains DNA (deoxyribonucleic acid). Muscle cells contain a number of nuclei and associate with muscle size (95). Satellite cells (SC) play a "navigation" role and distribute between sarcolemma and the basal membrane. When resistance training can result in a muscle damage, the SC will proliferate (i.e. multiple SC's). These new SC enter the cytoplasmic matrix and then begin to form new muscle fibers and additional nuclei. This potential mechanism was reported by Olsen, et al. (2006), who showed that Cr supplementation in combination with resistance training lead to a greater increase in the number of skeletal muscle SC and myonuclei concentration than resistance exercise alone (114). Although, conflicting findings exist to suggest that Cr supplementation may not improve muscle performance, strength, bone mineral content (98, 128), this may be explained by the dosage and duration Cr supplementation, or even potentially the variable baseline Cr status of muscle (32).

1.4. Role of Creatine in Skeletal Muscle Protein Turnover

1.4.1. Cellular Signaling Association with Skeletal Muscle Protein Turnover

Skeletal muscle mass is highly related to the net balance of muscle protein synthesis (MPS) and muscle protein breakdown (MPB) (64, 142). It has been well determined that skeletal muscle mass can be changed rapidly due to variety of stimulating factors, such as amino acids/protein intake, physical activities and nutrient availability (21). Previous research showed that amino acids/ protein ingestion can increase the amounts of substrate and signal for MPS, whereas other constituents of meals such as carbohydrate stimulate a slight inhibition of MPB (67, 76). These processes result in a positive net balance between MPS and MPB and overall muscle protein accretion. In addition to dietary consumption of amino acid/protein, muscle contraction (physical activities) induced muscle repair/regeneration is another stimuli of muscle protein turnover, especially following resistance exercise (RE) (59).

The Mammalian Target of Rapamycin (mTOR) which consist of two complexes (mTORC1 and mTORC2) is a key regulator of MPS (77, 136). This pathway is sensitive to amino acid (AA), cellular energy levels and growth factors and plays a key role on the regulation of protein translation and cell growth (90) in response to different types of mechanical stimulation (64). Whereas mTORC2 is sensitive to insulin and plays important role on regulating cytoskeleton, cell survival and insulin sensitivity (59, 90). To date, several target proteins has been well determined to involve in the regulation of mTOR signaling activity. One of the downstream targets of mTOR is the ribosomal protein S6 p70 Kinase 1 (p70S6K1) which, together with a upstream target Protein Kinase B (PKB or Akt), forms the Akt-mTOR-p70S6K signaling axis, which is involved in protein translation of mRNA (127, 183) and cell growth (90). The phosphorylation of S6K1 activates its kinase capacity and mediates phosphorylation of

ribosomal protein S6 to activate protein translation (76). Previous research from animal knockout models has elucidated that S6K1 is a key regulator of muscle hypertrophy. Another well-known mTORC1 target is the eukaryotic initiation factor 4E binding protein 1 (4E-BP1) (110, 125). This protein is inhibited and modulated by mTORC1 signaling pathway to suppress the translation initiation cap binding protein eIF4E. Intensive resistance exercise elevates 4E-BP1 phosphorylation and reduces the binding of 4E-BP1 and eIF4E, resulting in greater rates of translation initiation (52, 65). Alternatively, the Akt signaling pathway can modulate pro-line Akt substrate 40 kDa (PRAS40) function and may play a role in the inhibition of mTORC1 signaling (50). Ultimately, as MPS is a high energy demanding cellular process, it is not surprising that specific regulators exist to modulate mTOR signaling. Indeed, it is well described that mTORC1 signaling is repressed by AMP (Adenosine monophosphate)-activated protein kinase (AMPK) through the elevation of Tuberous Sclerosis Complex 2 (TSC2) activity (107).

1.4.2. Sensors of Cellular Energy Status

AMPK is an intracellular energy sensor and plays key roles in the regulation of anabolic and catabolic cellular processes and the modulation of exercise-induced adaptation (82, 143). AMPK responds to decreased cellular energy status and is activated in response to metabolic changes (164) in order to sustain energy homeostasis (31, 120). Protein synthesis requires four high-energy phosphates per peptide bond during the translation process (170). AMPK can move to the nucleus and modulate transcription status to promote energy production and meet cellular energy requirements (180). Alternatively, AMPK may also be involved in transport activity to modulate energy status (18).

Skeletal muscle AMPK signaling is activated in response to an increase in the ratio of AMP:ATP (120) that follows a reduction in ATP concentration (182). Activated AMPK signaling can inhibit cellular energy consuming processes, such as MPS (57, 182). It has also been demonstrated that the AMPK pathway can modulate glucose levels via glucose uptake processes. This indicates a potential association between AMPK and glucose transporter (GLUT4) activity (82), which is a key membrane transporter in skeletal muscle. GLUT4 plays a role in the transport of glucose into skeletal muscle in response to insulin production, mechanical loading and metabolic changes, thereby altering cellular energy status (135). Previously, Cr supplementation has been shown to increase resting muscle glycogen stores (9) and enhance muscle cell glucose utilization (80, 126) in human skeletal muscle. Cr participates in the energy production process together with glucose and mitochondria to generate ATP and meet cellular energy demands. Taken together, intramuscular Cr likely interacts with the other energy production systems to modulate intercellular energy homeostasis. As an energy modulator, AMPK responds to all the changes in energy status and, therefore, may also play roles in regulating intracellular Cr concentration via Cr uptake processes and may potentially up-regulate creatine transporter expression (CreaT) expression in skeletal muscle. Indeed, previous research showed that an elevation of intramuscular Cr: PCr ratio induced an activation of AMPK phosphorylation (99, 119). However, Cr or PCr concentration alone may not affect AMPK activity (120). Finally, AMPK has been shown in different levels of activation in different muscle fibre types (94). Specifically, Kirstensen et al. (2015) determined divergent AMPK responses accompanied different ratios of Cr: PCr in response to various types of exercise. Whereas the Cr: PCr ratio increased to a greater extent after interval (1.5 min cycling with 2.5 min interval) cycling exercise at ~100% V_{O2peak} compared with 30 min of continuous cycling exercise at ~70% of V_{O2peak} (28%) (94). The expression of Human skeletal muscle

α2β2γ3 AMPK complexes is lower in Type I fibre compared with Type II fibre (94). Due to the different pattern of fibre recruitment with different exercise forms, the different levels of AMPK expression may be associated with the intramuscular Cr uptake processes. To consider the function of CreaT protein in Cr metabolism, AMPK may play roles in regulating Cr uptake via modulation of CreaT expression, which has important implications for skeletal muscle energy metabolism, especially during intensive exercise. The potential signaling pathways of the interaction between cellular energy sensor status and Cr are displayed in **Figure 1.7**.

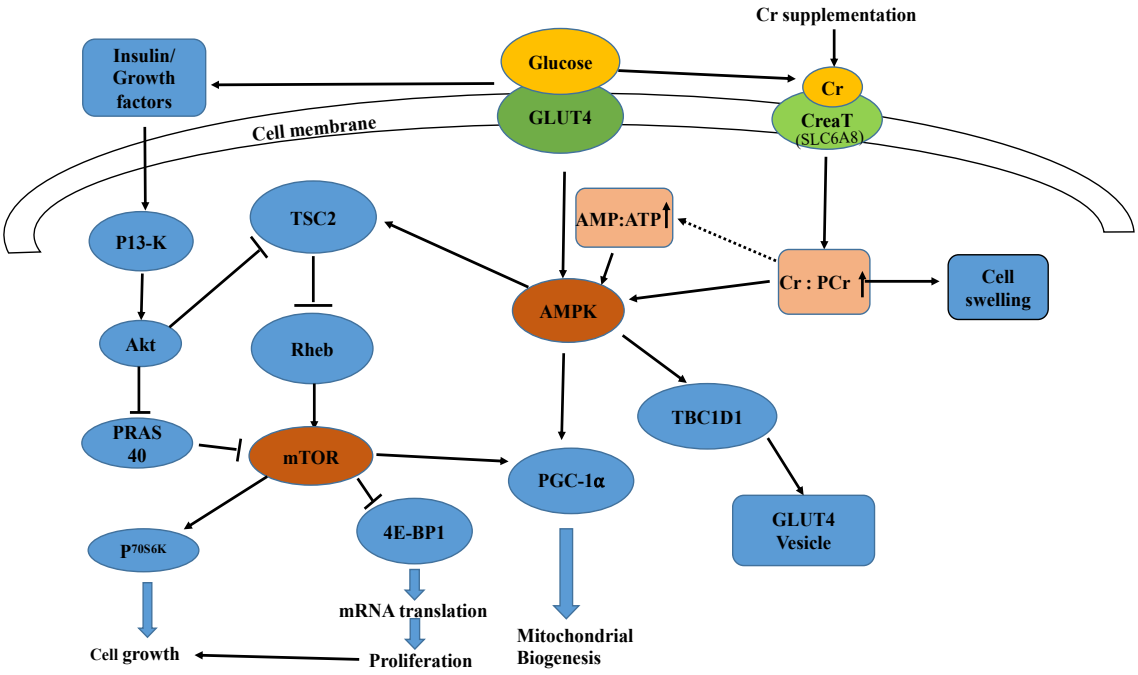

Figure 1.7 *Cellular signaling of energy sensor status and hypertrophy signaling. Following Cr supplementation, Cr is transported into skeletal muscle cell via CreaT protein and results in an increase of the ratio of Cr: PCr. High-intensity resistance training increases the ratio of Cr: PCr by decreasing PCr concentration and activating AMPK signaling to generate energy. P13-K: Phosphoinositide 3-kinase; Akt: Protein kinase B; PRAS40: Proline-rich AKT1 substrate 1; P^{70S6K}: P70-S6 Kinase 1; TSC2: Tuberous Sclerosis Complex 2; Rheb: Ras homolog; mTOR: The mammalian target of rapamycin; 4E-BP1: Eukaryotic translation initiation factor 4E-*

binding protein 1; GLUT4: Glucose transporter type 4; AMPK: 5' adenosine monophosphate-activated protein kinase; PGC-1a: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; TBC1D1: TBC1 Domain Family Member 1.

1.4.3. Interactions Between Creatine and Skeletal Muscle Protein Turnover

As mentioned in section 1.3.4, the enhancement in muscle mass with Cr supplementation during resistance training may be due to an augmented MPS response. This point of view has been reported in a number of studies, which showed Cr supplementation interacts with the proteins involved in the mTOR signaling pathway. Deldicque et al. (2005) reported that 5 days of high dosage (20g/day) of Cr supplementation intake combined with high intensity resistance exercise resulted in significant increases in 4E-BP1 phosphorylation in skeletal muscle (48). Burke et al. (2004) showed that 8 weeks of Cr supplementation in combination with resistance exercise increased intracellular insulin-like growth factor-1 (IGF-1) content by 67% (47). This was supported by Snow et al. (2008) and reported the Cr supplementation (0.4g/kg Cr + 0.4g/kg glucose) in combination with 65% V_{O2Peak} cycling exercise, led to the phosphorylation of proteins in the Akt signaling pathway (148). Safdar et al. (2008) reported that a short-term Cr supplementation increased Akt content (132). As mentioned in section 1.4.3, AMPK signaling is activated by alterations in the intramuscular Cr: PCr ratio. Cr supplementation has greater ability to increase cellular PCr levels than free Cr (53, 120). This unbalanced elevation result in an increased Cr: PCr ratio which may therefore activate AMPK signaling pathway and modulate mTOR signaling.

1.5. Creatine Transporter in Skeletal Muscle Cells

1.5.1. SLC6 Family and Creatine Transporters in Human

To date, creatine transport has been assigned to two different transporters; 1 and 2 (CreaT1 and CreaT2). It has been well studied that CreaT1 is associated with the SLC6A8 gene and mainly distributes in high energy demanding tissue such as the brain, heart and skeletal muscle. Whereas, CreaT2 found in kidney and retina (177). CreaT is one of the members in SLC6 family, particular in the γ-aminobutyric acid (GABA) transporter catalogue. All SLC6 members transport sodium/chloride ions together with their organic substrate to across cell membranes (130). In many cases, translocation of amino acids is against their concentration and coupled with transmembrane ion gradients. These ions can provide the energy to aid Cr access into muscle cells (129). The SLC6 family are tightly regulated by the interaction of other proteins (27), which leads to changes in transporter localisation. The changes of transporter localisation may occur via two potential mechanisms: firstly, binding with other proteins can result in movement of the transporter from intracellular membranes to cell surface; secondly, binding with other proteins can lead to stabilization and retention of the transporters at the cell surface (27, 49). The mechanism of membrane transport system is displayed in **Figure 1.8.**

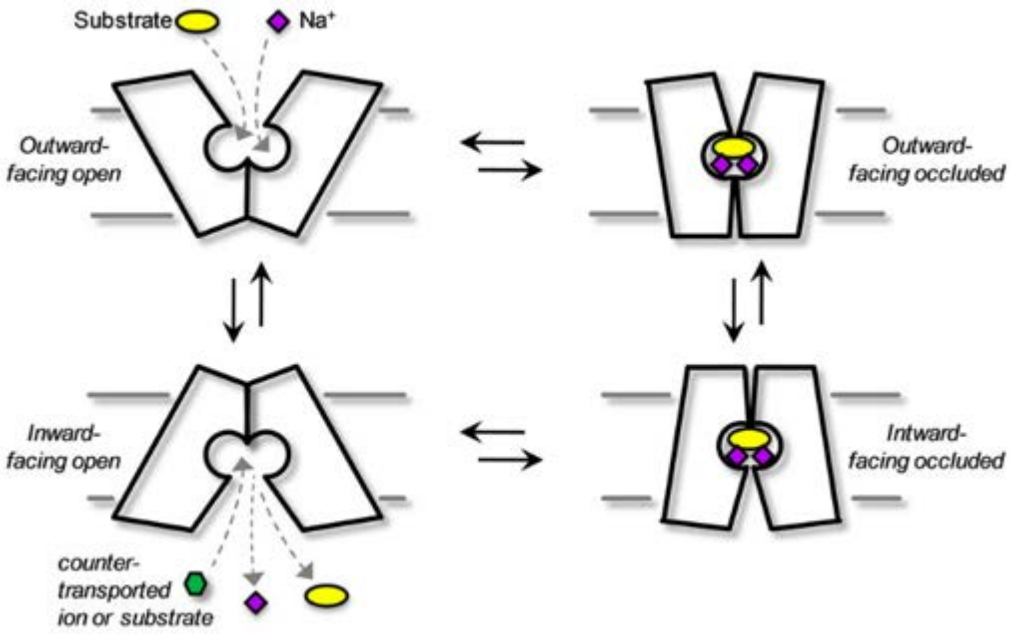

Figure 1.8 *Access mechanism of membrane transporters. The transporters work as a shuttle to transport substrate from extracellular space to the cytoplasm* (93).

1.5.2. The Role of CreaT in Cr Metabolism

CreaT1 (referred as CreaT) is currently the only known transporter protein of Cr, and is characterised as a highly-specific, saturable sodium/chloride (Na^+/Cl^-) gradient-dependent protein (146, 158), predominantly located at the sarcolemmal membrane in skeletal muscle. CreaT activity is important for skeletal muscle Cr uptake and metabolism (17). Indeed, muscle myopathies characterised by diminished intramuscular Cr and PCr have been linked to CreaT dysfunction (78, 159, 162). Similar to the function with other SLC6 transporter family members (38), CreaT activation is correlated with several different elements, such as extracellular and cytosolic Cr levels and the concentration of electric ions (117).

It has been well established that Cr uptake is a saturable process (167). In human skeletal muscle, the size of the Cr pool is relatively constant, whereas a reduction in intramuscular Cr concentration is associated with an increased content of CreaT protein (43, 146). Cr supplementation leads to a noticeable elevation of plasma Cr levels (147), and has been reported to significantly increase intramuscular TCr concentration. However, this elevation is not accompanied by altered CreaT mRNA and protein content in human skeletal muscle (158). As a specific transporter of Cr, CreaT protein expression should logically associate with cellular Cr/PCr levels. However, these divergent results indicate that other factors may affect the Cr uptake process. CreaT protein activity is highly associated with the electrochemical gradients Na^+ and Cl^-. Cr requires 2 Na^+ ions to drive the energy force to enter muscle cells (24). An insufficient concentration of ions potentially results in low Cr uptake kinetics via CreaT protein (3). A previous study found a replacement of NaCl gradient by a potassium or lithium chloride gradient in the sarcolemmal vesicles, completely abolished Cr transport (177) (**Figure 1.9**). Nevertheless, it has been demonstrated that CreaT needs only a relatively small portion of ions to enter muscle cells (25).

Recent research has indicated that skeletal muscle is able to synthesize very little Cr endogenously. Several studies have used CreaT knockout mice to investigate the contribution of CreaT to Cr uptake and muscle metabolism. in CreaT knockout mice, Skelton et al. (2011) identified reduced total body weight, undetectable brain and skeletal muscle Cr concentration, decreased heart, serum and testes Cr levels. Interestingly, the reduction in Cr content was not present in all organs of CreaT knockout mice, in particular the kidney which is the primary location of Cr synthesis (141). In line with Skelton's study, Russell et al. (2014) demonstrated a reduction of Cr content in CreaT knockout mouse model (131). This result suggest that skeletal muscle itself may able to produce a small amount of Cr endogenously (131). However,

the amount of Cr in CreaT knockout mice is insufficient to support normal tissue activities. Collectively, CreaT knockout mouse studies demonstrate that the essential and critical pathway for creatine transport is via its specific CreaT transporter. CreaT is therefore an irreplaceable component of Cr metabolism, with the absence of CreaT resulting in a decrease in TCr, PCr and ATP content as well impaired muscle mass and function.

In skeletal muscle, the distribution of CreaT protein content occurs in a fibre-type specific manner, with data demonstrating that the soleus and red gastrocnemius (greater proportion of Type I fibres) contain ~2-3 greater CreaT content than the white gastrocnemius muscle (greater proportion of Type II fibres) (146). These data suggest that Type I fibres may contain more CreaT than Type II fibres, a finding that has been confirmed in human muscle (109). Although, other potential signalling proteins, such as mTOR, AMPK, SGK1 (serum and glucocorticoid-regulated kinase 1 and), PGC- 1α and PGC-1 β (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha, 1-gamma) (30, 139) have been suggested to modulate CreaT mRNA, protein expression and activity, the potential inhibition and stimulatory factors involved in CreaT molecular biology (from transcription to translation) are yet to be well understood.

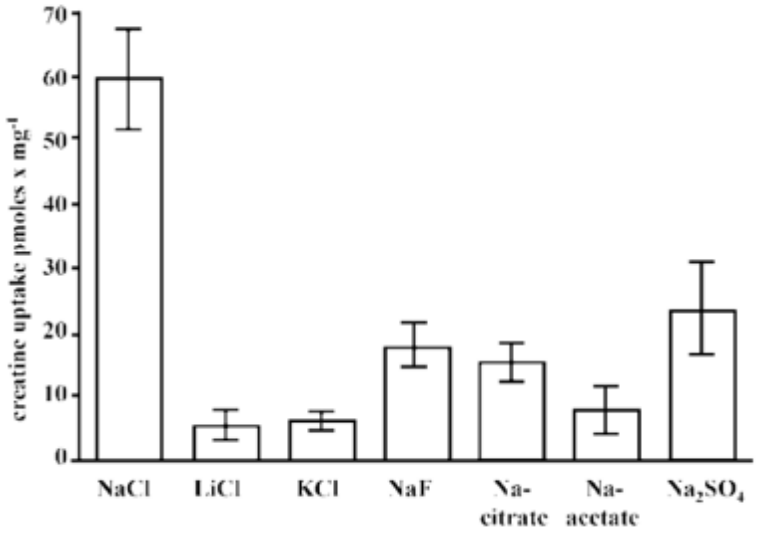

Figure 1.9 *Effect of ions in Cr transport procedure. Cr transport is considerably diminished when uptake buffers containing ions other than* Cl^- *were used. Exchange of* Na^+ *lead to a significant loss of Cr transport activity* (177).

1.5.3. Capillary Recruitment in Skeletal Muscle

Blood vessels are an important part of the circulatory system, transporting blood throughout body. There are three main types of blood vessels, including arteries, capillaries and veins. The arteries export blood from heart, capillaries exchange water and chemicals between cell tissues and blood and the veins return the blood from the capillaries to heart. Nitric oxide (NO) is a small gaseous and lipophilic molecule which involves in various biological processes as an universal mediator, such as neurotransmission via different pathways (40, 63, 169). Endothelial nitric oxide synthase (eNOS) is an enzyme which participants in the synthesis of nitric oxide (NO) and predominantly activate the generation of NO in the vascular endothelium (58). Skeletal muscle is perfused by a complex network of capillaries, delivery oxygen and substrates to working cells, simultaneously eliminating metabolic wastes. Cr are transported to high energy demanded tissues via this blood vessels network. Previous studies reported that Cr uptake displayed at conditionally immortalized mouse brain capillary endothelial cells (TM-BBB), which is Na^+ and Cl^- dependent (113). CreaT is expressed in TM-BBB cells and isolated mouse brain micro-vessels (113). Furthermore, high expression of CreaT was observed in the mouse brain capillaries by confocal immunofluorescent microscopy (113). Additionally, GABA transporter were reported to localize at blood-brain barrier (BBB) (156). These results imply that CreaT plays an important role in supplying Cr with a high efficiency of Cr transport at blood–tissue junctions.

1.6. Impaired Creatine Metabolism in Disease

Cr homeostasis is influenced by several factors, such as endogenous Cr synthesis and degradation, dietary Cr ingestion and the efficiency of Cr uptake in tissues. Impaired Cr hemostasis is associated with various type of diseases, which especially affect the functions of high-energy demand organs, such as the brain and skeletal muscle. Insufficient Cr will cause an abnormal energy homeostasis and disturbed cellular functional. Endogenous Cr deficiency disorders are induced by mutation in AGAT, GAMT and SLC6A8 (51). These patients typically suffer from intellectual disabilities, a delay of language development, mental retardation and behavioural abnormalities (137). GAMT deficiency and AGAT deficiency can be treated by Cr supplementation (55). Whereas, SLC6A8 deficiency is thought to be difficult or impossible to treat with Cr supplementation (123). This is due to the important function of CreaT in Cr metabolism, with the loss of this transport carrier resulting in a barrier to Cr entry into cells.

Despite mutation-related deficiency disorders, myopathies also interact with abnormal Cr homeostasis (160). Patients suffering from myopathies such as Hereditary muscle disease, Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD), spinal muscle atrophy (SMA), or diabetic myopathy suffer from progressive weakness by impaired energy regulation, leading to contractile failure (i.e., mitochondrial myopathy, McArdle's disease) (159, 161). In addition, Tarnopolsky et al. reported reduced CreaT mRNA and possibly protein content in myopathies compared with a healthy population (162). Furthermore, DMD disturbs homeostatic concentration of ions (177), as the function of the CreaT protein is highly dependent on the concentration of the sodium and chloride gradient. Impaired ion homeostasis may reduce Cr uptake rates and therefore decrease the intramuscular TCr pool. Other neurological disorders such as Parkinson's disease and Alzheimer's disease have been reported

to associate with abnormal Cr metabolism (160). This is not surprising, as Cr functions to energy generating processes to sustain cellular activities. Overall, balanced Cr metabolism is important for patients suffering from different diseases to attenuate the loss of muscle mass and functional disability, and this appears to be highly dependent on CreaT expression and activity.

1.7. Muscular Ageing and Disuse

1.7.1. Ageing-Induced Changes in Skeletal Muscle

Ageing-related loss of skeletal muscle mass and strength (terms as sarcopenia) leads to a reduction of muscle function and performance, increased risk disability and mortality (42, 104). Currently, these aged-related skeletal muscle changes impact over 50 million people and for the next 30 years will rise dramatically, affecting more than 200 million people worldwide (44, 96). The predicted climate of geriatric syndromes will place a significant stress on our healthcare resources (5). As such, the development of appropriate therapeutic interventions to counteract age-related musculoskeletal deterioration is paramount. Previous studies found that the age-related loss of muscle mass occurs relative of fibre-specific changes (111, 122), with a reduction in total number of both Type I and Type II fibres and a predominant atrophy of Type II fibres (122, 138). Type II muscle atrophy in ageing results in lager percentage of slow twitch Type I muscle, as evidenced by slower contraction times in older muscle (138).

Aged-related loss of muscle mass reflects a progressive decline in anabolism and increase in catabolism, alongside with a reduced muscle regenerative capacity (111). Previous studies have highlighted that there are several potential mechanisms that drive sarcopenia progression, which include impairments in MPS (179), alterations of nutrition intake (83, 101), hormonal impairments (111), inflammatory changes (111) and reductions in physical activity (16, 68,

179). Loss of muscle mass is in response to negative energy balance are due to the imbalanced rate of MPS and MPB (41, 83). Previous studies showed that MPS declines by ~30% with ageing (68), whereas almost no changes of MPB (11), which lead to negative net balance of protein turnover. Imbalanced protein turnover will disrupt protein homeostasis, which refers to proteostasis. Proteostasis is generally related to the location, conformation, and turnover of individual proteins (12). Impaired proteostasis result in the accretion of damaged proteins. In skeletal muscle, accretion of these damage protein could result in the reduction of quantity and quality of contractile proteins. Protein turnover is important to sustain muscle proteome. Improvement of proteostasis is associated with the increased net balance of protein turnover. This improvement is achieved by regeneration new, functional proteins and degrading damaged proteins. Protein turnover is a high energy-consuming process (127, 143). Protein synthesis requires 12–72 ATP molecules for each amino acid to be synthesised (12), whereas the energetic cost of protein breakdown has yet to be clearly understood. Nonetheless, in order to meet the energy requirement of protein turnover, a consistent source of energetic production is fundamental to sustain proteostasis.

Cr supplementation has been shown to augment muscle mass and strength with ageing and other diseases, with well-defined benefits for skeletal muscle performance and the health of other tissues, such as the brain (35, 144). Intracellular Cr levels in older individuals are ~25% lower than younger adults (28, 145). Generally, individuals with lower intramuscular TCr concentrations display a greater ability to enhance intracellular Cr with supplementation (72). Therefore, it is logical to expect that older adults (with lower intramuscular TCr) could expect a significant benefit from Cr supplementation combined with resistance exercise than younger individuals (with higher intramuscular TCr). Indeed, a 12-week resistance training study in older adults showed an enhancement of lean muscle mass accretion with Cr supplementation

(118). Similarly, Tarnopolsky reported a significant increase in fat-free mass and muscle strength following 6 month resistance exercise combined with Cr supplementation with in older men and women (65-85 yrs) (157). Brose et al reported that a combination of Cr supplementation and resistance exercise enhanced intramuscular PCr and TCr concentration, fat-free mass, isometric knee extension strength and muscle function in older men and women following 14 weeks intervention (28). The mechanisms through which Cr supplementation augments resistance exercise-induced skeletal muscle remodeling and enhances cellular Cr concentration have yet to be fully understood. In skeletal muscle, the Cr-PCr system has been shown to transfer high-energy phosphates from mitochondria to cytoplasm via various types of CK (4, 108). This is the major contributor to meet energy requirement of muscle contraction, as the regeneration of PCr will increase ADP concentration for mitochondrial respiration (175). Increased cellular Cr concentration after Cr supplementation intake may provide sufficient Cr and increase the capability of energy transfer from mitochondria to working tissues. Therefore, this may explain the beneficial effects of Cr supplementation in supporting skeletal muscle mass remodeling with exercise training. As CreaT is currently the only known transporter protein of Cr and appears to be essential in skeletal muscle Cr uptake and metabolism (17), alterations of CreaT may have implications for aged-related muscle loss via maintenance of proteostasis.

1.7.2 Disuse-Induced Muscle Atrophy

Muscle atrophy is initiated by several causes, including progressive ageing (81, 154), muscle disuse (173) and disease (152). Loss of muscle mass leads to a reduction in muscle strength and function. Short periods of muscle unloading (i.e. during hospitalization or immobilization) results in a considerable loss of skeletal muscle mass and strength, which is generally

accompanied by a lengthy recover time following re-ambulation, a higher risk of injury and impaired metabolic health (97, 168). Therefore, short periods of immobilization have important clinical relevance, and effective strategies are required to attenuate the loss of muscle mass and function during short periods muscle disuse.

Evidences suggests that disuse-induced muscle atrophy is potentially due to a negative net balance of muscle protein turnover (171). The mechanical unloading of skeletal muscle significantly impairs cellular homeostasis, which result in an initiation of muscle remodelling processes (171). Previously, it has been demonstrated that just 5-days of leg immobilization diminished fasted and fed-state MPS rates in healthy young individuals. Accordingly, it is clear that disuse induced skeletal muscle atrophy in humans is driven by diminishing of both postabsorptive and postprandial MPS (77, 116). In contrast to the impairment of MPS during muscle disuse, the role of MPB in disuse induced atrophy is unclear. Studies have suggested that MPB does not change during prolonged bed rest (116, 171). Furthermore, others have suggested that the changes in muscle protein turnover may occur rapidly after the onset of disuse (i.e. the first few days) whilst the muscle attempts to restore homeostasis (171).

Short-term disuse-induced muscle atrophy is accompanied with reduction of high-energy phosphagens in muscle (79, 106). Cr supplementation has been shown to attenuate the reduction of intramuscular PCr concentration during seven days of cast-immobilization (61), in line with suggestions that Cr supplementation may counteract muscle atrophy during disuse (91). Several studies even suggest that Cr supplementation may potentially be more effective in scenarios of disuse (73) and disease (100, 103) induced muscle deterioration. Specifically, Johnston et al. showed that Cr supplementation protected muscle mass and strength compared with a placebo with 21 days of immobilization in humans (84). In rats, Aoki et al. also reported that 14 days of Cr supplementation (5g/kg over 7 days prior-to and during 7 days of immobilization)

attenuated the loss of muscle mass in soleus and gastrocnemius muscles in rats (102). Collectively, these findings lend support to the effectiveness of Cr supplementation as a strategy to protect against disuse-induced muscle deterioration. Furthermore, it is apparent that alterations in intramuscular Cr and/or PCr may play a role in disuse-induced muscle atrophy. However, the relative mechanistic link between alterations in muscle morphology and Cr metabolism during disuse are yet to be understood. As a specific transporter of Cr, CreaT expression and sub-localisation may have implications for disuse-induced muscle atrophy. Specifically, muscle unloading results in a declines in energy requirements and appetite/hunger (172). One hypothesis is that low cellular energy status during disuse may initiate AMPK signaling as a means to preserve cellular ATP and energy homeostasis (18, 180), but also inhibit the mTOR signalling pathway. In support of this notion, Darrabie et al. reported that AMPK activation increased the amount of CreaT protein localisation at the cell surface in cardiomyocytes (46). Furthermore, Cr supplementation has been shown to increase cellular energy status and decrease glycolysis via inhibition of AMPK pathway in chicken pectoralis muscle (184). On the basis of the existing evidence, alterations in cellular energy status appear to be associated with AMPK activation. Therefore, CreaT expression and distribution may implicated in muscle atrophy during disuse events.

1.8. Overview and Knowledge Gaps

CreaT is integral for Cr entry into skeletal muscle cells and plays key roles in regulating cellular Cr metabolism. As a part of energy generation system, sufficient Cr is essential to maintain energy homeostasis (either independently generates ATP or interact with mitochondria and glucose to generate ATP). It has been well established that a number of cellular metabolic processes require a large amount of energy, including muscle protein turnover. A negative net balance of protein turnover leads to the reduction of muscle mass, with implications for strength, function and numerous comorbidities. Therefore, the maintenance of intramuscular Cr is important for sustainability of skeletal muscle mass across the life-course. Loss of skeletal muscle can be initiated by various reasons, such as progressive ageing and disuse (i.e. during illness or injury) and disease. Muscle atrophy conditions are associated with the decline in intramuscular high energy phosphagens, whereas Cr supplementation has the potential to increase muscle mass and strength in the elderly and attenuate disuse-induced muscle atrophy. Collectively, the weight of evidence supports the key role of Cr metabolism in the maintenance of muscle health. As such, investigations of CreaT protein expression and sub-cellular localisation are vital in order to enhance our understanding of the role of Cr in skeletal muscle physiology. Alterations in CreaT may explain muscle deterioration in conditions of ageing and disuse atrophy, and therapeutic interventions to support muscle health may well need to be targeted to CreaT. However, the role of CreaT in modulating Cr metabolism in skeletal muscle remain largely unexplored. To date, measurements of CreaT have been conducted through traditional methods to determine gene expression (mRNA) and protein content in muscle homogenates, or relatively basic microscopy techniques. As CreaT protein only functions at cell membrane, existing measurement techniques may lack of precision to fully explain the regulatory role of CreaT in different physiological scenarios of muscle deterioration.

1.9 Aim and Hypotheses

Based on the established knowledge gaps, the first aim of this thesis was to develop a sophisticated immunofluorescence microscopy technique to study CreaT expression and localisation in human skeletal muscle (**Chapter 2**). We hypothesized that our newly developed technique would reveal more accurate information on skeletal muscle CreaT than previous studies including, for the first time, data to show that CreaT co-localized predominantly to cell membranes. By using this newly developed immunofluorescence microscopy technique, the second aim of this thesis was to investigate the CreaT expression and sub-cellular localisation in skeletal muscle from healthy young and older individuals (**Chapter 3**). We hypothesized that skeletal muscle CreaT protein expression would be reduced in aged skeletal muscle, particularly at the cell periphery, thereby offering a potential explanation for age-related differences in Cr metabolism. The third aim of this thesis was to investigate the effect of immobilization on CreaT protein expression and sub-cellular localisation in human skeletal muscle (**Chapter 4**). We hypothesized that, alongside reductions in muscle mass and strength, CreaT protein expression and localisation at the cell periphery would be lower in muscle that had undergone 7-days of immobilization compared with the contralateral non-immobilized control limb, allied to observations from others that disuse results in a diminution of muscle PCr.

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Chapter 2

General Methods and Method Development

2.1 Introduction

Skeletal muscle CreaT expression is associated with intramuscular Cr concentration which, in turn, is uniquely sensitive to resistance exercise and Cr supplementation consumption. Previous data suggest that changes in cellular energy status through exercise and/or Cr supplementation intake may initiate signalling pathways which stimulate CreaT mRNA expression and upregulate CreaT protein content in human skeletal muscle (1). However, it is difficult to link the physiological relevance of changes in CreaT protein content with CreaT activity since CreaT is thought to function at the cell membrane (4, 23). Therefore, the measurement of CreaT mRNA and protein content in a whole muscle homogenates may lack of precision to explain CreaT biology and the CreaT response to changes in cellular energy status in different physiological scenarios. Immunofluorescence microscopy methods could therefore be used to provide stronger evidence to determine CreaT content, sub-cellular localisation and functionality. However, previous studies investigating CreaT protein have been limited due to the low expression levels of the CreaT and/or a lack of specificity/sensitivity of CreaT antibodies (6, 18, 21). Previously, an immunofluorescence microscopy method was development and used to detect CreaT activities in neurons of rat and human tissues (7, 14, 15). However, little is known about CreaT biology in skeletal muscle tissue, which is the predominant site of creatine storage in the human body. Murphy et al. were the first to use an immunofluorescence microscopy technique to visualize the intramuscular location of CreaT protein in rat skeletal muscle, and determined that CreaT was predominately localized at cell membrane, with some signal also present throughout the rest of the cell (16). Following this technique, the same group conducted a human study and reported a muscle fibre-type specific expression of CreaT protein content (17). Important to note is that ‘cocktail’ antibodies (mixed CreaT and MHCI) were used in this study, which may have influenced the intensity of the

fluorescent signal and adversely affected the findings. Furthermore, although negative controls (without secondary antibodies) were applied, the typical weakness of most commercially available CreaT antibodies means that other critical antibody controls need to be implemented (i.e. primary antibodies controls between CreaT and MHCI), to avoid unreliable conclusions. Therefore, the aim of the present chapter was to validate and optimize a contemporary sophisticated immunofluorescence microscopy technique to study fibre-type CreaT content and sub-cellular localisation in human skeletal muscle.

2.2 Ethical Approval

Human skeletal muscle samples used in Chapters 2-4 were obtained from School of Sport, Exercise and Rehabilitation Sciences, University of Birmingham. Ethical approval for the collection of muscle samples was granted by the NHS West Midlands Black Country and Solihull Research Ethics Committees (13/WM/042, 15/WM/0228 and 17/EM/0086) and conformed to the principles of the Declaration of Helsinki (7^{th} version). Written informed consent was obtained from all participants before samples were collected.

2.3 Human Skeletal Muscle Collection and Preparation

2.3.1 Sample Collection

Skeletal muscle samples used for immunofluorescence histological microscopy and western blot method development were obtained from the *vastus lateralis* of a sub-set of six healthy human individuals. Muscle tissues samples were collected by using the Bergstrom percutaneous needle biopsy technique, under local anaesthesia (Bergstrom, 1975). Samples were cleaned by using sterile saline to remove excess blood and dissected visible fat and collagen. Biopsy tissue

was then snap frozen in liquid nitrogen for western blot or placed in Tissue-Tek OCT compound (Sakura, 4583) and frozen in liquid nitrogen-cooled isopentane for immunofluorescence microscopy (Sigma Aldrich, 270342) before being stored at -80°C until later analysis.

2.3.2 Sample Preparation for Immunofluorescence Staining

Embedded muscle samples were fixed in front of a microtome blade (Bright 5040, Bright Company limited, Huntingdon, England) by Tissue-Tec OCT under -25°C temperature. An appropriate angle and position were adjusted before sectioning. Following adjustment cryo-sections (10 µm) were collected onto glass microscope slides (VWR International) and left in room temperature for 30 min to remove excess crystallized water before stored in the -80 °C freezer. Slow steady rotation of the hand wheel was required while cutting sections to ensure the sections were kept flat and smooth. This was important to ensure consistent quality of muscle sections for subsequent steps of staining.

2.3.3 Sample Preparation for Western Blot

Snap-frozen skeletal muscle samples of ~30 mg, were powdered on dry ice by using a pestle and mortar, homogenised buffer (50 mM Tris-HCL, 1mM Ethylene glycol tetra-acetic acid (EGTA), 1 mM Ethylenediaminetetraacetic acid (EDTA), 10 mM β-Glycerophosphate, 50 mM NaF) with a Protease Inhibitor Tablet (Roche No. 05892791001) and 0.5mM sodium orthovanadate was added to the muscle sample. Then the muscle tissue was placed in eppendorf tubes and kept on ice. Approximately 300µl lysis buffer was rapidly added into the tube and homogenized with an electric homogenizer. This step was repeated for three times; 70% ethanol and Millipore water were applied between repeats to keep the blade in a clean condition. The volumes of lysis buffer were determined by the amount of tissue present. Then the homogenate

was left on ice for 10 min before being vortexed. Samples were centrifuged at 8000xg 10 min at 4°C temperature and the supernatant was aspirated and placed in a new eppendorf tube kept on ice. A detergent compatible (*DC™)* protein assay was used to determine protein concentration, the protein standards were diluted by using a serial dilution with bovine serum albumin (BSA) and lysis buffer. 100ul of 4 × Laemmli buffer (Bio-Rad) was used to minimize dilution of the samples together with an appropriate volume of homogenised buffer (dilute samples at 20×) added to samples before boiling at 95 ℃ temperature for 5 min. All samples were normalised at a protein concentration of 2 $\mu g/\mu l$.

2.4 Enzymatic Analysis

Skeletal muscle biopsy samples were cleaned to remove macroscopic blood and dissected free of any visible fat and collagen. Then approximately 10mg of freeze-dried skeletal muscle tissues was powdered, and metabolites extracted in 0.5 M perchloric acid (containing 1 mM EDTA), followed by neutralisation with 2.2M K2CO3 (Sigma Aldrich, Gillingham, UK). Cr, PCr and ATP were determined enzymatically in muscle extracts according to the method of Harris et al, modified to accommodate use of a 96-well plate spectrophotometer (9). TCr is reported as the sum of PCr and Cr. Metabolite contents were corrected for non-muscle constituents by using muscle ATP content.

2.5 Immunoblotting

2.5.1 Immunoblotting Protocol

An equal amount of protein extract (20ug) was loaded and separated on 10% acrylamide gels by SDS-PAGE for approximately 1 h at 23 mA/gel with 5 µl molecular weight markers (Bio-

Rad, UK). A pooled sample was used as a gel control to ensure all wells were loaded with same amount of protein. Proteins were then transferred to a biotrace nitrocellulose membrane (Pall Laboratory, Portsmouth, U.K.) for 1h at 100 V in 1x Tris/Glycine buffer with 20 % methanol. The transfer duration was dependent on the molecular weight of protein, with larger proteins normally involving a lengthier transfer period time. Membranes were subsequently stained with Ponceau S solution and washed 4-5 times with water (until there were no traces of Ponceau in the water and all lanes were clearly defined) and imaged immediately to validate the transfer of protein from each lane. Then membranes were blocked in 5% skim dry milk in TBST (Cell Signalling, USA) to prevent potential non-specific background binding. After blocking, membranes were incubated overnight at 4°C in anti-Creatine transporter (CreaT) antibody (ab62196) (1:1000 in Tris-buffer saline, with 0.1% Tween (TBST)) (same antibody used in immunofluorescence staining), and anti-Creatine Kinase (CK) MM antibody (ab54637). In the peptide blocking experiment, anti-SLC6A8 antibody (abcam, ab62196) was pre-incubated with its corresponding peptide (abcam, ab216175) (5 times greater than primary antibody concentration) at 4 ℃ overnight. Membranes were then washed 3 × 5 min with TBST, and incubated in goat anti-rabbit IgG Horseradish peroxidase (HRP) conjugated secondary (Thermo Fisher, cat.31460, 1:10000 in TBST) for 2 h at room temperature before washing in TBST and detected by Immobilon Western chemiluminescent HRP substrate (Merck Millipore, Watford, UK). Images were captured by using a G:BOX Chemi XT4 imager with GeneSys capture software (Syngene, Cambridge, U.K.). A Chemi Genius Bioimaging Gel Doc System (Syngene) was used to image capture and bands quantification. Ponceau S was used as a loading control to identify that the gel lanes were loaded with sample, and used as a comparison to determine the protein expression levels in different samples. Proteins were expressed relative to Ponceau

S to verify the efficiency of the current western blot protocol, and specificity of the antibody on binding with the target protein, which may not be present in the experimental samples.

2.5.2 Antibody Optimization

Immunoblotting was used to identify the antibody expressed a particular single protein at the expected molecular weight (2) and used as a comparable evidence to determine total skeletal muscle CreaT content. CK concentration was optimized by different dilutions and the amount of protein loading. Antibodies used in western blot are listed in **Table 2.1**. All general procedures for western blotting in this thesis were the same.

Table 2.1 *Summary of antibodies used for western blot*

Primary antibody	Source	Dilution	Secondary antibody	Dilution
Polyclonal anti-SLC6A8 antibody with rabbit antigen, IsoType IgG	abcam, ab62196	1:400	Anti-rabbit HRP-linked antibody IgG 7074S	1:10000
SLC6A8 peptide	abcam, ab216175	1:80	Anti-rabbit HRP-linked antibody IgG 7074S	1:10000
Monoclonal anti-Creatine Kinase MM antibody with mouse antigen, IsoType IgG1	abcam, ab54637	1:8000	Anti-mouse HRP-linked antibody IgG 7076S	1:10000

2.6 CreaT Immunofluorescence Microscopy Method Development

The CreaT protein immunofluorescence staining protocol was optimised by determining immunogen peptide control, different fixation solutions, antibody dilutions, primary and secondary antibody control experiments to produce the optimal images and quantifiable data. The entire optimised protocol is outlined in detail in section 2.6.1. The immunofluorescence staining protocol for analysis of CreaT in human skeletal muscle was optimized by using negative controls, this was performed to ensure the specificity of the CreaT protein antibody. Pre-cut cryo-sections were removed from the freezer ~15 min before fixation in order to remove any ice or water on the surface of sections.

2.6.1 CreaT Immunofluorescence Staining Protocol

All primary antibodies, secondary antibodies, blocking peptides and experimental dilutions used for immunofluorescence staining in this thesis are listed in **Table 2.2**.

2.6.1.1 CreaT Co-stain with Dystrophin

Sections were fixed in 4% paraformaldehyde (PFA) solution (Fisher, UK) for 10 min at room temperature. Samples were then washed for 3× 5 min in phosphate buffered saline (PBS) with 0.2% Tween (PBS-T) to remove the fixation reagent before blocking with 5% normal goat serum (NGS) (Invitrogen, UK) in 1% bovine serum albumin solution (1% BSA) for 1 h at room temperature. In blocking peptide experiments, the anti-SLC6A8 antibody (ab62196, abcam, Cambridge, UK) was pre-incubated with its corresponding peptide (ab216175) (at 5 x greater than the primary antibody concentration) for 1 h at room temperature. Following the blocking stage, primary antibody CreaT (goat-anti-rabbit IgGα, 1:150, abcam, Cambridge, UK) and

dystrophin (mouse IgGα, 1:100) were incubated at 4℃ overnight. Sections were washed 3 × 5 min following primary antibody incubation and then incubated with secondary antibodies for 2 h at room temperature. Following secondary antibody incubation, muscle sections were washed with PBST 2 × 5 min and PBS 1 × 5 min to remove the remaining secondary antibodies. Sections were then left to dry for ~15 min at room temperature and mounted with 10ul ProLong® Gold Antifade Mountant (P36930, Invitrogen™, UK,) and covered with glass coverslips to protect the sections and maintain optimal conditions for fluorescence signals. Slides were left to dry overnight in a dark place before capturing images.

2.6.1.2 CreaT Co-stain with eNOS

Sections were fixed with acetone to ethanol mixed solution in a 3:1 dilution (Fisher, UK) for 5 min at room temperature. Samples were then washed for 3× 5 min in phosphate buffered saline (1×PBS) with 0.2% Tween (PBS-T) to remove the fixation reagent before blocking with 5% normal goat serum (NGS) (Invitrogen, UK) in 1% bovine serum albumin solution (1% BSA) for 1 h at room temperature. Following the blocking stage, primary antibody CreaT (goat-anti-rabbit IgGα, 1:200, abcam, Cambridge, UK) and eNOS (mouse IgGα, 1:200) were incubated at 4℃ overnight. Sections were washed for 3 × 5 min following primary antibody incubation and then incubated with secondary antibodies (goat-anti-mouse IgG1 594, 1:200 and got anti-rabbit, 1:200 in 1×PBS) for 2 h at room temperature. Following secondary antibody incubation, muscle sections were washed with PBST 3× 5 min to remove the remaining secondary antibodies and then incubated with WGA (1:20) at room temperature for 30 min. WGA was used to mark cell borders. Then sections were washed with PBST for 2× 5min and 1× 5min PBS following WGA incubation. Slides were then left to dry for about 15 min at room temperature and mounted with 10ul ProLong® Gold Antifade Mountant (P36930, Invitrogen™, UK,) and covered with glass

coverslips to protect the sections and maintain optimal conditions for fluorescence signals. Slides were left to dry overnight in a dark place before capturing images.

2.6.1.3 Skeletal Muscle Fibre-type Staining

Sections were incubated with Triton X 100 (0.02%) for 5 min at room temperature to identify skeletal muscle fibre type distribution. Samples then washed 3 ×5 min in phosphate buffered saline (PBS) with 0.2% Tween (PBS-T) to remove the fixation reagent. Sections were then blocked in 5% normal goat serum (Invitrogen, UK) in 1 × PBS for 90 min at room temperature. Following blocking stage, sections were incubated with primary antibodies MHCI (IgG2b, BAF8, DSHB, Iowa, US) and MHCII (IgG1, SC.71, DSHB, Iowa, US) at room temperature overnight. On day two, sections were washed for 3 × 5 min in 1 × PBS-T at room temperature after primary antibody incubation. Secondary antibodies were then applied to sections for 90 min to detect fibre type distribution at room temperature. Wheat germ agglutinin (WGA) was used in skeletal muscle fibre type distribution experiments to mark sarcolemmal membranes. Following secondary antibody incubation, sections were then washed for 2 × 5 min with PBS-T and 1 × 5 min with PBS and left to dry for ~15 min. In the end, sections were mounted with 10ul ProLong® Gold Antifade Mountant (P36930, Invitrogen™, UK,) and covered with glass coverslips to protect the sections and to maintain optimal conditions for fluorescence signals. Slides were left to dry overnight in a dark place before capturing images.

Table 2.2 *Summary of antibodies used in immunofluorescence microscopy*

Primary antibody	Source	Dilution	Secondary antibody	Dilution
Polyclonal anti-SLC6A8 antibody with rabbit antigen, IsoType IgG	abcam, ab62196	1:150	Goat anti-rabbit IgG Alexa®488	1:200
SLC6A8 peptide	abcam, ab216175	1:40	Goat anti-rabbit IgG Alexa®488	1:200
Monoclonal anti-Dystrophin antibody with mouse antigen, IsoType IgG2a	DSHB, MANDYS1 3B7	1:100	Goat anti-mouse IgG2a Alexa®594	1:200
Purified Mouse Anti-eNOS/NOS Type III	BD Transduction	1:200	Goat anti-mouse IgG1 Alexa®594	1:200
Monoclonal anti-MHC1 antibody with mouse antigen, IsoType IgG2b	DSHB, BAF8	1:25	Goat anti-mouse IgG2b Alexa®546	1:100
Monoclonal anti-MHCII antibody with mouse antigen, IsoType IgG1	SC-71	1:20	Goat anti-mouse IgG1 Alexa®488	1:100
Wheat Germ Agglutinin-350	W11263, Invitrogen	1:50	Alexa Fluor® 350 Conjugated	N/A

2.6.2 Antibody Validation

The accuracy of immunofluorescence is dependent on the specificity of the primary antibody that is only targeted to the desired protein of interest. In this thesis, a number of systematic control experiments were combined to determine immunofluorescence staining specificity and to identify ideal conditions of the target proteins in human skeletal muscle. The antibody control tests included peptide blocking, primary and secondary antibodies controls. Theoretically, numerous methods could be used to determine the specificity of an antibody (3), knockout animal models, mass spectrometry, expression profiles (online) and peptide blocking are all widely used methods to determine antibody specificity, and each method has advantages and disadvantages. For example, knockout animal models are a very powerful antibody validation technique and typically guarantee no/limited expression of the target gene or protein. Nevertheless, animal facilities and expertise access are required in this method, these can be quite time-consuming and expensive to generate. Fortunately, we were able to obtain muscle tissue samples from wild-type and CreaT KO mice, kindly donated by Professor Rod Snow (Deakin University, Australia), used in their groups earlier work (20). CreaT KO tissue allowed us to generate a direct validation of our CreaT antibody specificity (in mixed muscle homogenates only, as no sections were available). In addition, peptide blocking was also used here as a reliable method for antibody validation/specificity, with the advantage that only a small volume of sample required.

2.6.2.1 CreaT Protein Antibody Peptide Blocking

In this study, CreaT antibody and its immunogen peptide control test were carried out to confirm antibody specificity and selectivity in an immunofluorescence microscopy experiment.

As peptide immunogens have the advantage of generating antibodies for a single isoform of a protein, and is therefore appropriate to validate the CreaT antibody used in this thesis. The anti-SLC6A8 antibody (ab62196, abcam, Cambridge, UK) was pre-incubated with its corresponding peptide (ab216175) together on a saturating concentration (5 times greater than the primary antibody concentration) for 1 h at room temperature. The remaining stages of peptide control experiments were as same as the CreaT co-staining with dystrophin experimental procedures described above.

2.6.2.2 Primary Antibody Optimization

To confirm the most appropriate concentration of CreaT primary antibody, a series of dilutions were performed under the same staining procedures and same concentration of secondary antibody. The superior dilution was selected based on the highest signal, lowest background noise level and the lowest amount of antibody usage. The concentration of dilution was based on recommendations from the antibody manual guide. The CreaT protein antibody was diluted with the recommended concentration (1:200) and compared with 1:100 and 1:150 dilution. Furthermore, the specificity of protein stains was validated by negative control staining. Phosphate buffered saline (1xPBS) was used to dilute antibodies, reagents and washing steps. The incubation condition of the primary antibody was 4°C overnight.

2.6.2.3 Secondary Antibody

By considering species cross-reactivity, 5% normal goat serum was incubated with muscle sections before secondary antibody incubation to reduce potential unspecific binding from secondary antibodies. All secondary antibodies used in this thesis were conjugated with Alexa@ series fluorophores (Invitrogen, Paisley, UK) unless specifically mentioned. When the

experiment has several different proteins co-stained together, secondary antibodies targeting different proteins were detected by different Alexa dye fluorophores. For examples, CreaT primary antibody was distinguished by secondary antibody conjugated with Alexa 488 (Green stain), Dystrophin primary antibody was detected by secondary antibody conjugated with Alexa 594 (Red stain). Validation experiments were also developed to prevent potential cross-binding between secondary antibodies during co-stain experiments.

2.6.2.4 CreaT Co-stain with MHCI

In this thesis, cross-binding controls have been applied when performing multiple antibody stain. The validation of CreaT antibody specificity has been described in previous paragraphs. Therefore, in this section, a cross-binding control test was based on the staining of CreaT. This cross-binding control has been performed by controlling of MHCI primary antibody and secondary antibody. Sections were divided into two different groups, both of which were incubated with CreaT primary antibody and homologous secondary antibody in the first instance. In these control experiments, group one was applied to identify non-specific binding of the MHCI primary antibody. Group two was applied to determine the effects of MHCI primary and secondary antibody staining on potential binding with the CreaT stain. Following CreaT primary antibody incubation, in first group, section one was incubated with both primary and secondary antibody, however, section two was incubated with MHCI primary antibody but without secondary antibody. In group two, section one was incubated with MHCI primary antibody and corresponding secondary antibody. Section two was simply incubated with 5% GS in PBS instead of MHCI primary antibody and incubated with PBS to take the place of its corresponding secondary antibody. The remaining steps of the immunofluorescence staining protocol were processed by following the protocol in section 2.6.1.1 and the competition

staining which was finalized together with the positive controls. The specificity of the antibodies targeting MHCI and MHCII to identify muscle fibre-type (22, 24), dystrophin and WGA to mark the cell membrane (12) have all been confirmed in earlier studies.

2.6.3 Antibody Control

For each tissue staining protocol, certain controls were carried out (5). PBS with 5% normal goat serum was applied to the tissues instead of primary antibody to make sure no tissue autofluorescence or no-specific secondary antibody binding with muscles sections. When no positive staining visualised on sections, non-autofluorescence or non-specific secondary antibody bind were confirmed.

Immunoblot was used as a primary antibody control to determine the specificity of the primary antibody at the correct molecular weight. The protocol of immunoblot has been described in previous paragraph (2.5).

To ensure no cross-reactions and appropriate labelling, controls were performed in which only one primary antibody was applied to the tissue with all secondary antibodies. When the signal was observed only with its corresponding secondary antibody, the antibody was confirmed as targeting to its primary antibody with no cross-reactions arising.

To confirm each fluorophore only released a signal that was detected in a single channel, controls were carried out in which only one target was stained. Images were captured in all channels to confirm that signal was only detected in the channel specific to the target stain.

2.6.4 Fixation Optimization

An appropriate fixation is essential for immunofluorescence staining. In this thesis, fixation method, time and temperature were considered in optimizing immunofluorescence protocol.

4% formaldehyde (PFA) was used in the immunofluorescence staining as a fixation solution. This fixation method results in low levels of shrinkage and good preservation of cellular structure for a wide range of cells and tissues and does not cause substantial structural changes to proteins. Triton X-100 was used in immunofluorescence detection of muscle fibre types.

2.6.5 Image Capture

Wide-field image capture was completed by using Nikon E600 with a 40×0.75 numerical aperture objective. Images per area were captured under three colour filters achieved by a SPOTRT KE colour shot CCD camera (Diagnostic Instruments Inc., MI, USA), illuminated by a 170 W Xenon light source. For image capture, the Texas-Red (540-580mm) excitation filter was used to capture signals of dystrophin (sarcolemma) or MHCI (Type I fibres), which were conjugated with Alexa Fluor 546 or 594 fluorophores and CreaT or MHCII (Type II fibres) stains tagged with Alexa 488 fluorophore (green) visualised under the FITC (465–495 nm) excitation filter. DAPI UV (340–380 nm) filter was used to view WGA-350 (blue) signals.

2.6.6 Image Analysis

All microscope images were processed under grey/white raw image format. The association between CreaT and cellular organelles was measured by co-localisation. The subcellular distribution of CreaT was measured by quantification analysis.

In co-localisation analysis, approximately seven regions per section were randomly selected and captured under same microscope setting. Images were processed and analysed under the Image-Pro Plus 5.1 software (Media Cybernetics, MD., USA.) and Image J software. Image signals from WGA and Dystrophin were used to evaluate cell membrane borders, then merged with the corresponding target proteins images to classify the correlation between proteins and

the sarcolemmal membrane. Pearson's correlation coefficient was used to measure co-localisation of target proteins and Dystrophin in immunofluorescence images. Background images were captured per section, CreaT content intensity and co-localisation experiment data were analysed by excluding background noise. All images analysis methods were kept consistent within the same study.

2.6.7 Image Quantitation Analysis

Each slide contained two young and two old skeletal muscle biopsy sample sections, a total of three slides replicates, were stained, imaged and quantified. On average, approximately eight images were captured per section, and each capture contained at least eight muscle fibres. Approximately 80 fibres per subject were used for analysis. During quantification, background images were taken from each section to lower the background noise. CreaT fluorescence intensity was quantified by measuring the signal intensity within the intracellular regions of a mask created by the dystrophin stain in a fibre type specific manner as determined from the MHCI stain.

2.6.8 Calculation

Ring area mean integrate density was used to identify CreaT protein content intensity around cell membranes. Image J software was used to ensure the enlarged inner CSA was consistently in each cell. First of all, cell membranes have been circled along the cell membrane (which determined by dystrophin) to get outbound CSA (named as A) and integrate density values (named as B), then the circles were enlarged with a fixed distance (-10 pixel units) to get the inner CSA (named as A') and integrate density values (named as B'). This fixed distance was selected following a series of selection tests. By using the Image J software, the enlarged

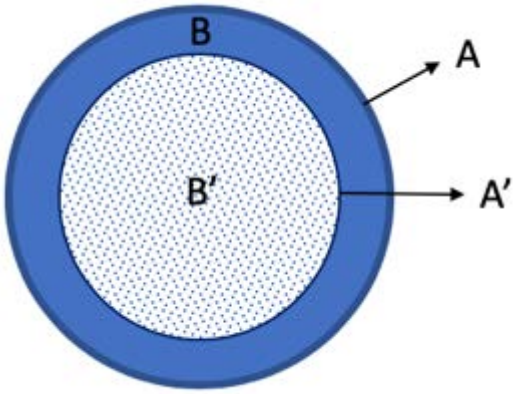
distance has been selected as 1 pixel units, 2 pixel units, 5 pixel units, 10 pixel units, 15 pixel units and 20 pixel units in 6 different sections. Each section contains at least 7 muscle fibres (at 40 X magnification). 10 pixel units were selected for all the analysis of localisation in this thesis. This is due to this fixed distance is appropriated to just include all the spots clusters around cell membranes and not too far away from cell membranes. The ring area was identified as inner CSA subtracted from outbound CSA (A- A') and ring area integrate density was identified as inner CSA intensity subtracted from outbound CSA integrate density (B- B'). Then mean ring area intensity was calculated by ring area integrate density (B- B') divided by ring area (A- A'). CreaT protein expression has been indicated by using this method. CreaT intensity and density are present by using a relative unit of measurement to show the ratio of amount of substance, refer as arbitrary unit (a.u. or AU). The calculations are listed below:

Raw integrate density (RawIntDen) = Sum of pixel values in selection

Integrate density = RawIntDen * (Area in scaled units)

Mean intensity = integrate density / area in pixels

$$\text{Ring area mean intensiy} = \frac{(\text{B} - \text{B}')}{(\text{A} - \text{A}')}$$



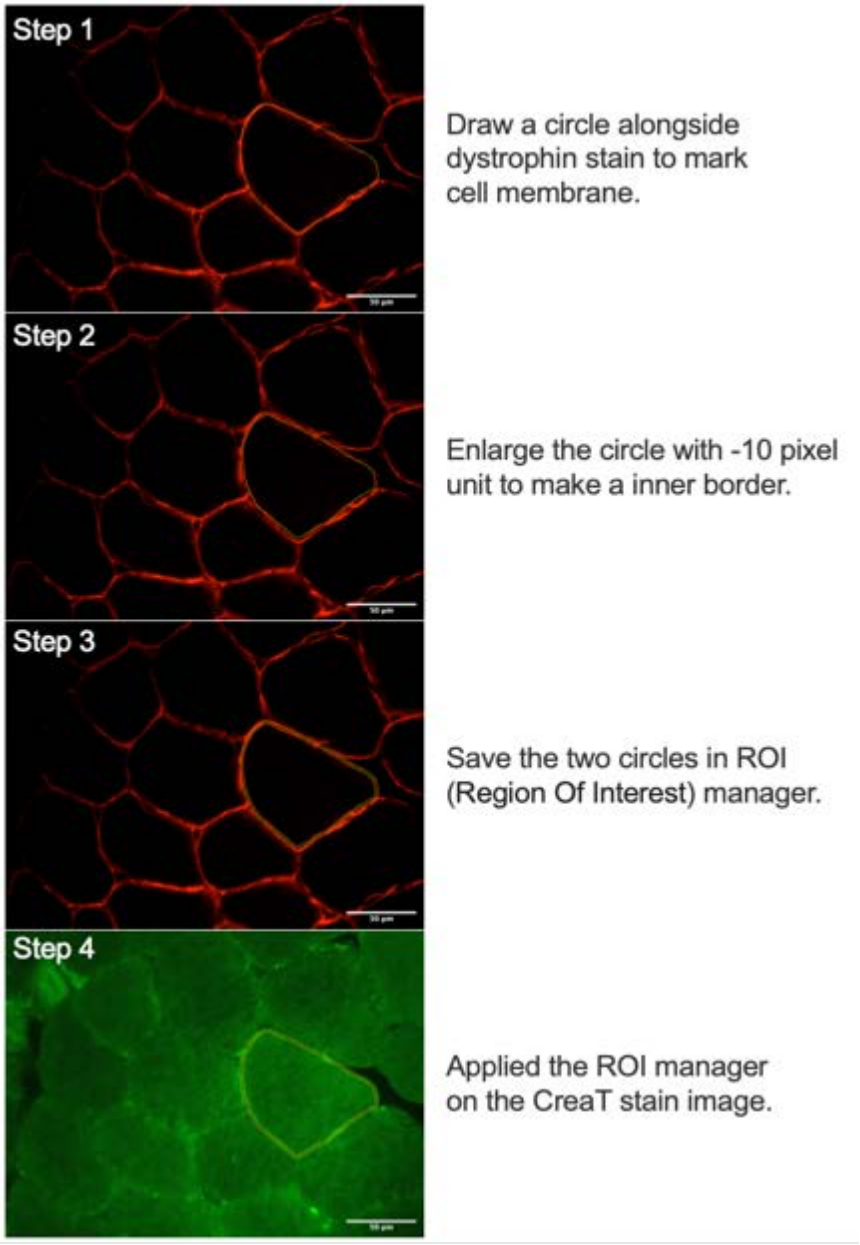


Figure 2.1 Illustration of the steps involved in Ring area calculation.

2.6.9 Statistics

Repeated immunofluorescence images quantification and colocalisation analysis were applied to ensure the repeatability of antibodies from same muscle samples before formal studies. The repeated stain and measurement for specific antibody was evaluated by the range of coefficient of variation (CV) between duplicates. In experimental chapters, all immunofluorescence

imaged quantification was performed in duplicate (2 sections of same sample on 1 slide). The data for each subject was the mean value of two duplicate sections on one slide. For statistical analysis, Pearson's correlation coefficient values and protein fluorophore intensity obtained from seven to eight areas per section were input into statistics software SPSS, version 22. An unpaired T-test was used to identify the significant difference of data at same time point between different groups. A paired t-test was used to determine Type I and Type II muscle fibres in same subject. Repeated measurements were used in multi-comparison conditions (i.e. fibre types × specific cellular regions). Significant difference was determined by $P < 0.05$. Data was presented as Mean ± SD. GraphPad Prism was used to present figures and tables (GraphPad Prism for Mac version, San Diego, California).

2.7 Results of CreaT Antibody Optimization

2.7.1 Antibody Validation

In human skeletal muscle samples, a positive CreaT protein stain was observed in the centre area and the ring area (green stain). The intensity of the CreaT stains were brighter around ring area (cell membranes) than the central area stain (**Figure 2.2A**). Incubation of the CreaT primary antibody with the immunogen blocking peptide prior to application to the muscle section, almost completely abolished CreaT staining intensity in all cell regions, leaving only background fluorescence and confirming specificity of the antibody (**Figure 2.2B**, **Figure 2.2C**). Western blotting (**Figure 2.2D**) was applied to human skeletal muscle to confirm the specificity of CreaT primary antibody, which should only detect the antibody in a single band at the specified molecular weight (~70 kDa). Exclusion of the CreaT primary antibody resulted in no staining, confirming that the secondary antibody specific bound to the target protein

without any tissue autofluorescence. Additionally, not only the peptide blocking experiments applied to validate the specificity of CreaT antibody. CreaT protein Knockout (KO) and wild type (WT) mice tissue were used to investigate the specificity of CreaT protein antibody, results showed that KO mice tissue did not present bands on membrane where the WT tissues display faint but detectable bands around 70 KDa (**Figure 2.3**). Taken together, these data suggested that the anti-CreaT primary antibody (ab62196, abcam, Cambridge, UK) used in the current study is specific to the CreaT protein in human skeletal muscle.

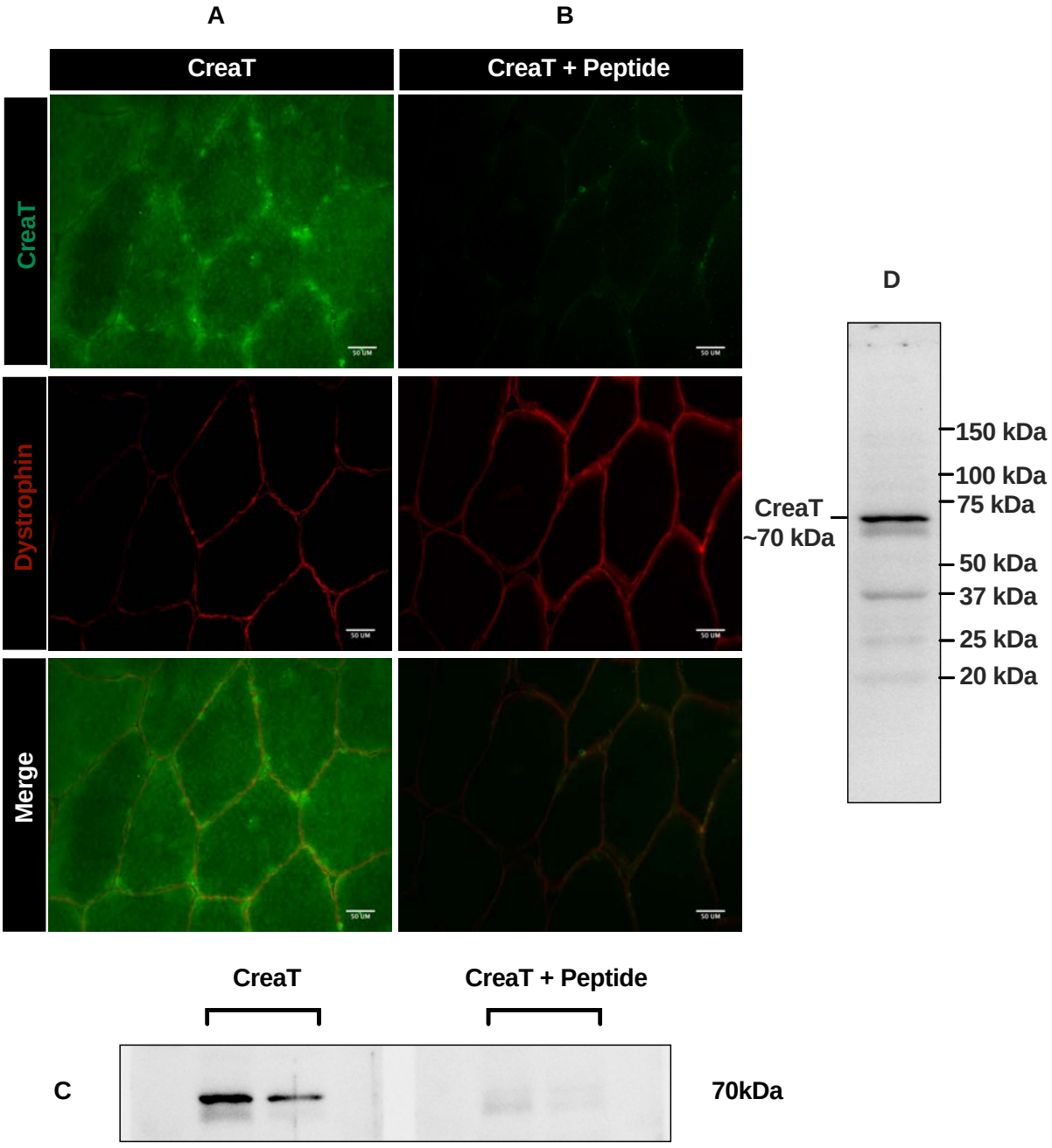


Figure 2.2 *Immunofluorescent and Immunoblotting CreaT peptide blocking. Human skeletal muscle marked with CreaT (green stain) to detect CreaT protein, Dystrophin (Red stain) to mark sarcolemma.* ***A.*** *CreaT antibody only.* ***B.*** *CreaT antibody with its peptide blocking.* ***C****. CreaT peptide blocking in western blot, the two bands on left side are CreaT antibody only, and the other two bands on right side are CreaT with peptide.* ***D****. Human skeletal muscle CreaT protein Western blot.*

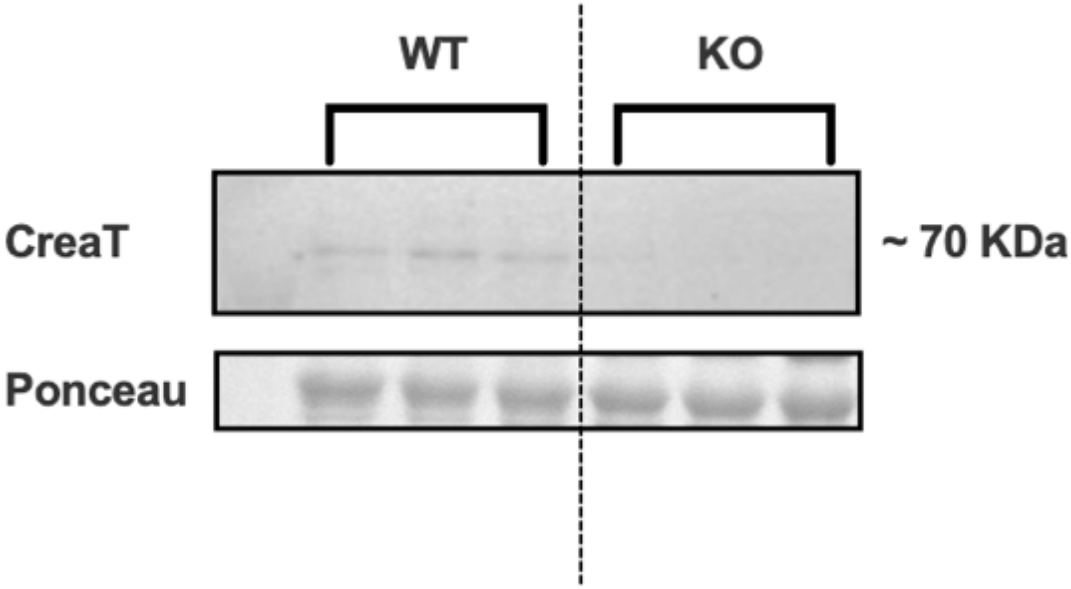


Figure 2.3 *Detection of CreaT KO and WT mice skeletal muscle. KO (n=3) and wild type (n=3).*

Validation of CreaT primary antibody concentration.

CreaT primary antibody was serially diluted at 1:100,1:150 and 1:200. All secondary antibodies were diluted by 1:200. The best dilution was determined by the signal strength, the background noise and the amount of antibody usage. The images in **Figure 2.4** showed that a 1:150 primary body dilution was the best of three due to its lower background noise, the visualised signal and the lower antibody usage.

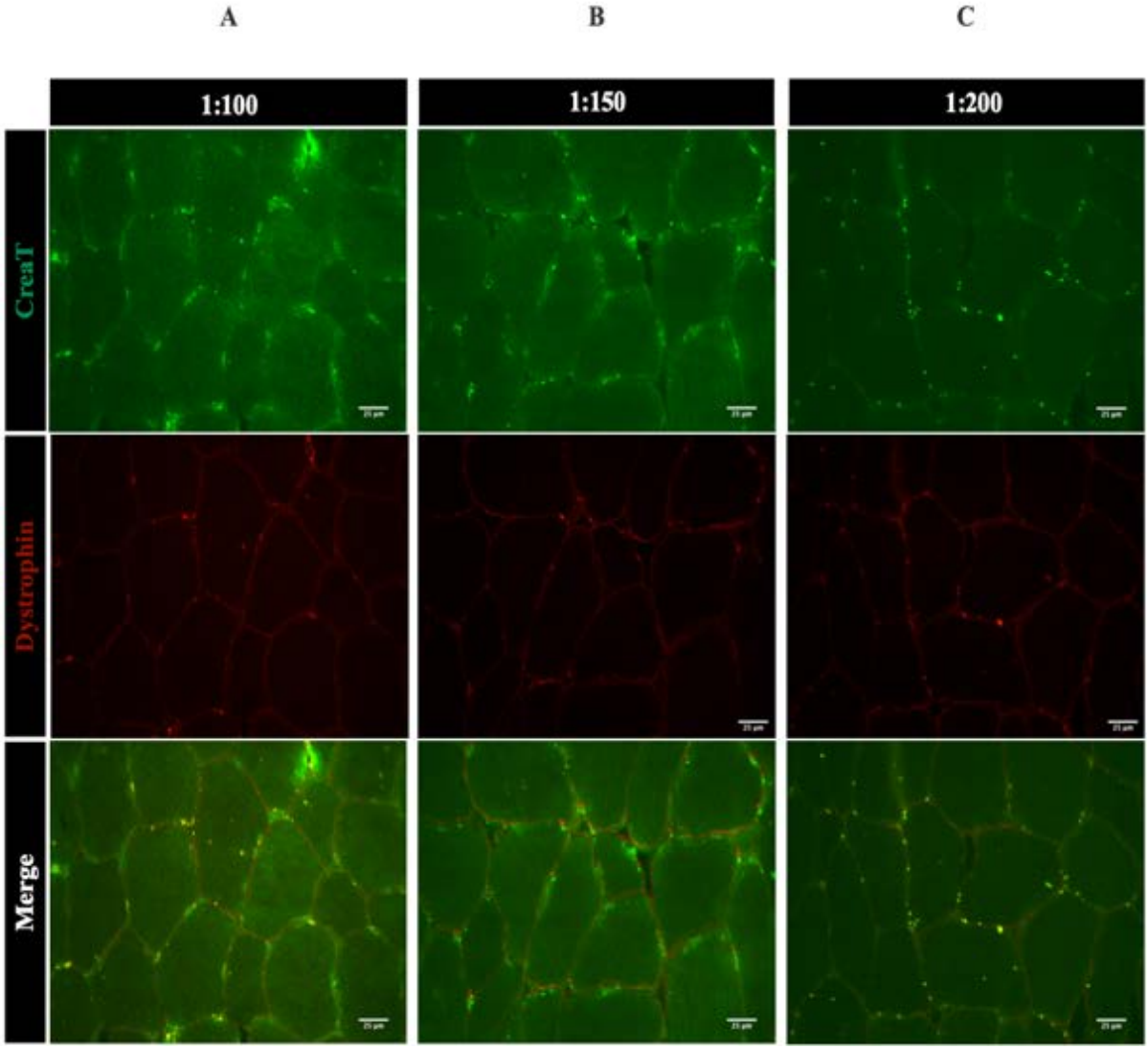


Figure 2.4 *CreaT antibody dilution. Human skeletal muscle marked with CreaT (green stain) to detect CreaT protein, Dystrophin (Red stain) to mark sarcolemma. A is CreaT antibody diluted at 1:100, B is CreaT antibody diluted at 1:150, C is CreaT antibody diluted at 1:200.*

A CreaT primary antibody negative control (no CreaT primary antibody but a secondary antibody co-stain with dystrophin primary and secondary antibodies) was applied to ensure the exclusive of unspecific fluorescence of the primary antibody. The results of this experiment are shown in **Figure 2.5**, suggesting that the CreaT primary antibody was specific to the target.

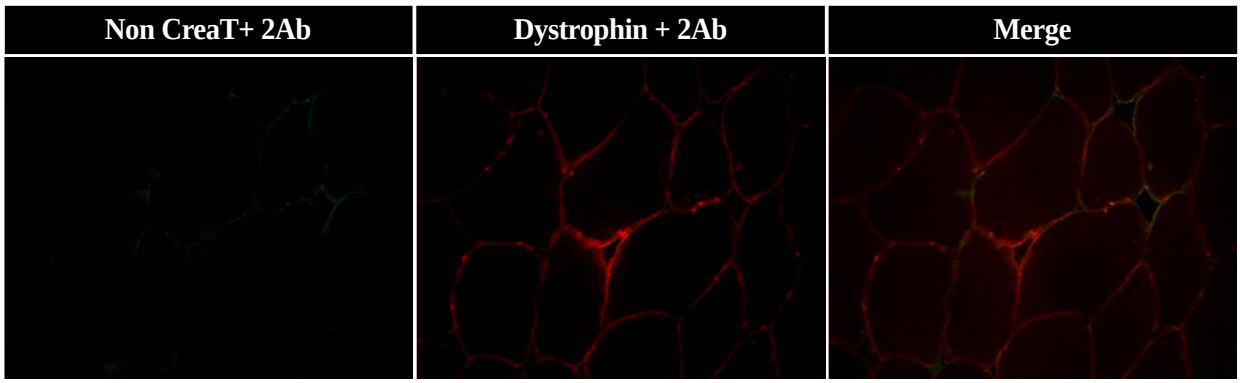


Figure 2.5 *Antibody controls. Sections stained only with the secondary antibody of CreaT. Human skeletal muscle marked with dystrophin (Red stain) to mark sarcolemma.*

2.7.2 Basal CreaT Intensity and Co-Localisation in Human Skeletal Muscle

CreaT staining in human skeletal muscle appears as large clusters at the cell periphery with smaller spots spread throughout the rest of the cell, as can be seen in **Figure 2.6**. This was demonstrated through CreaT co-stain with the cell membrane marker dystrophin. The localisation of CreaT in human skeletal muscle shown throughout this thesis revealed that CreaT was mainly associated with the cell membranes in all muscle fibre-types, similar to observations in previous studies (16).

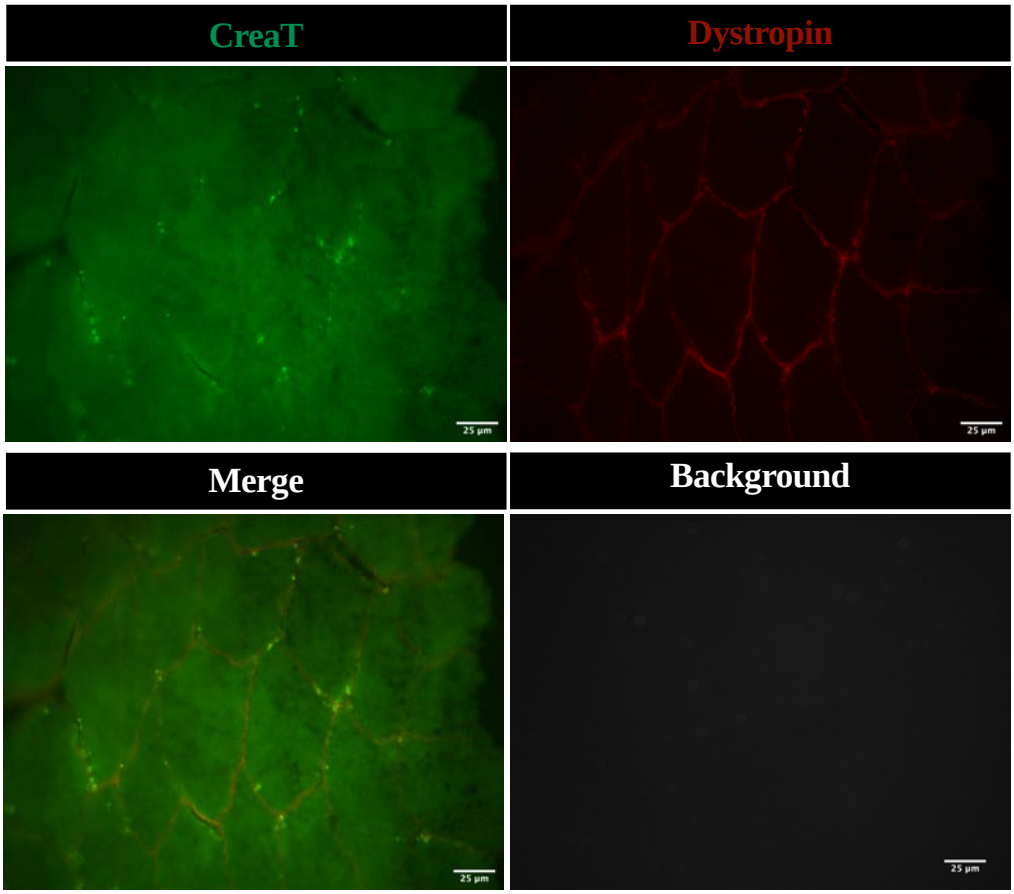


Figure 2.6 *Immunofluorescent stain of CreaT and Dystrophin. Human skeletal muscle marked with CreaT (green stain) to detect CreaT protein, Dystrophin (Red stain) to mark cell membrane. All images subtracted their background intensity when calculated CreaT intensity to decrease the effect of background noise.*

2.7.3 CreaT Co-stain with MHCI

In group one, the section was incubated with both primary and secondary (CreaT co-stain with MHCI). Greater CreaT protein was observed in Type I compared with Type II fibres (shown in **Figure 2.7A**). In the CreaT stained section excluding the MHCI primary antibody, the signal intensity was less bright (**Figure 2.7B**). In group two, section one was incubated with both primary and secondary (CreaT co-stain with MHCI) **(Figure 2.8A)**. Exclusion of MHCI and its

corresponding secondary antibody showed the same result with control test 1B (**Figure 2.8**B), (The section stains with CreaT primary + CreaT corresponding secondary + MHCI corresponding secondary antibody) confirming that the secondary antibody did not react with CreaT antibodies. Taken together, the results of the two cross-binding control experiments determined that the MHCI primary antibody potentially results in non-specific staining that caused a higher fluorescence signal, which may impact the quantification of CreaT intensity. Considering the cross-binding effects between antibodies, these results suggest that when CreaT is co-stained with MHCI, the intensity would be greater than CreaT protein stained alone. Therefore, in this thesis, when MHCI staining was required, it was completed on different sections to CreaT to avoid co-staining.

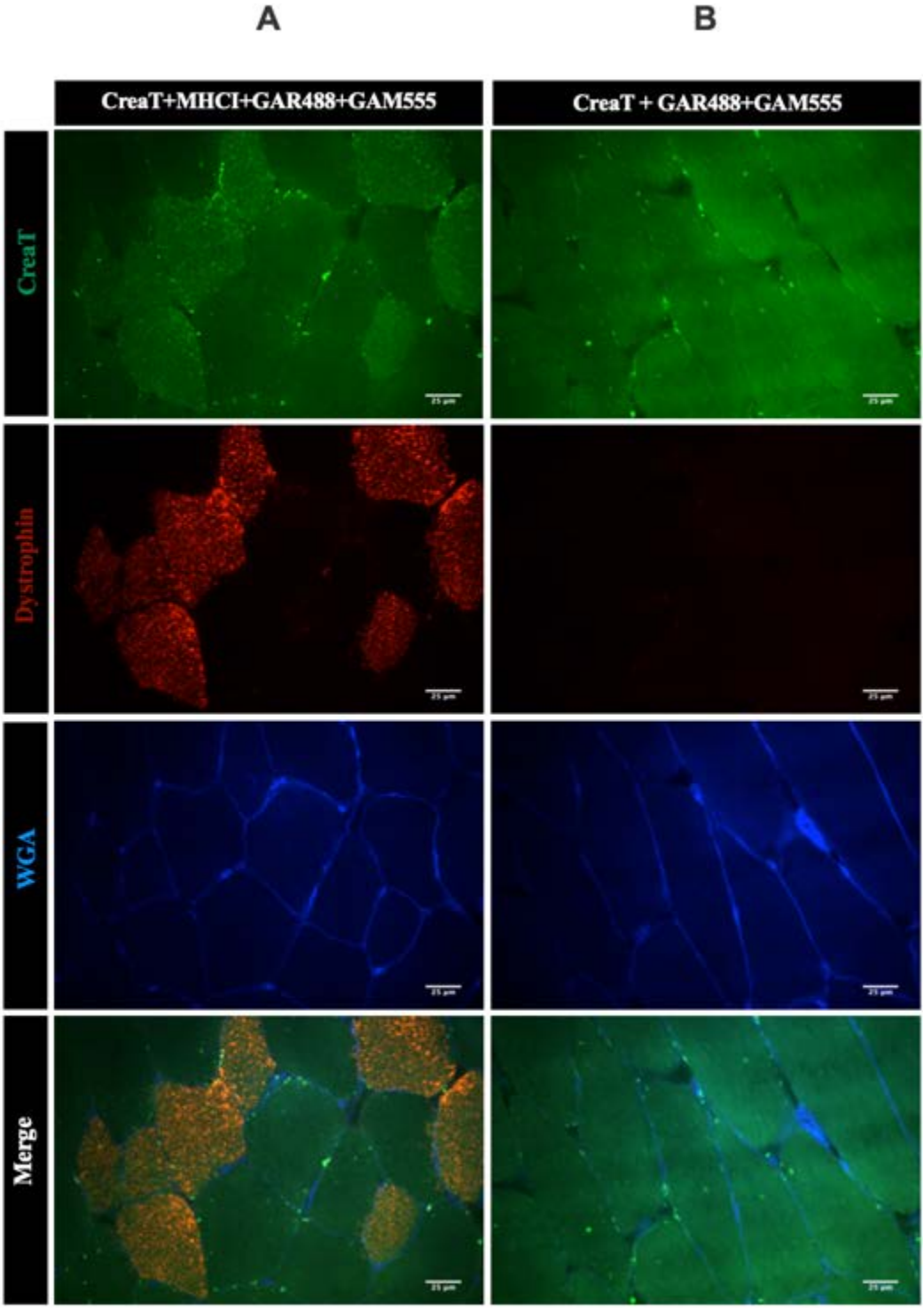


Figure 2.7 *Immunofluorescent CreaT co-stain MHCI control test 1. Human skeletal muscle marked with CreaT (green stain) to detect CreaT protein, MHCI (Red stain) to determine Type I muscle fibres and wheat germ agglutinin (WGA) to mark cell membranes.* ***A****. CreaT and MHCI primary*

antibody with corresponding secondary antibodies; ***B****. CreaT antibody without MHCI primary antibody but with both secondary antibodies.*

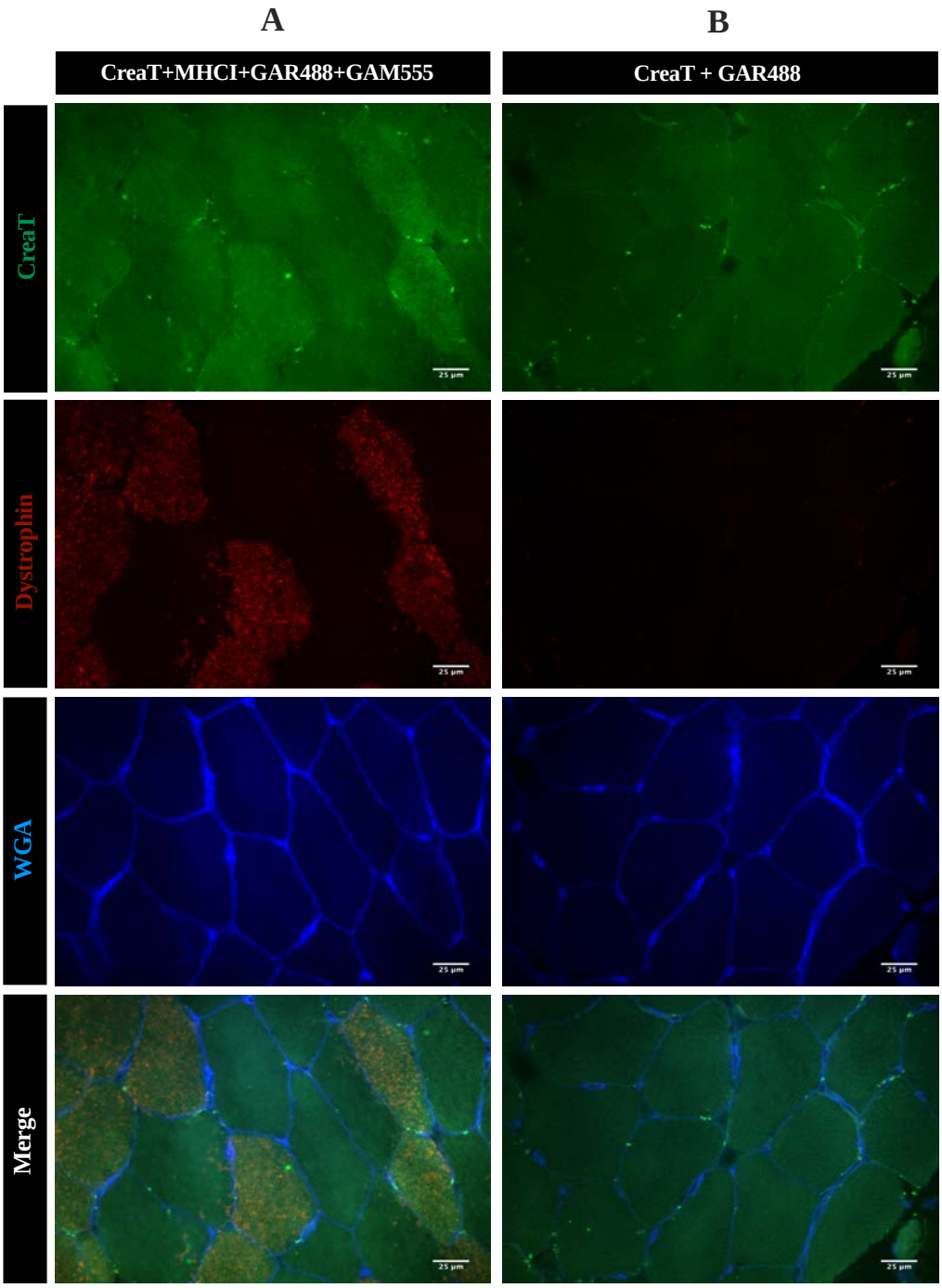


Figure 2.8 *Immunofluorescent CreaT co-stain with MHCI control test 2. Human skeletal muscle marked with CreaT (green stain) to detect CreaT protein, MHCI (Red stain) to determine Type I muscle fibres and wheat germ agglutinin (WGA) to mark cell membranes.* ***A****. CreaT and MHCI*

primary antibody with their secondary antibodies. ***B****. CreaT primary antibody with its secondary antibody.*

2.7.4 Human Skeletal Muscle Fibre-type Distribution and Cross Section Area (CSA)

Human skeletal muscle fibre-type distribution was determined by immunofluorescence staining of muscle cross section area (CSA) of Type I and Type II fibres (**Figure 2.9**C). MHCI (red stain) expressed Type I muscle fibres and MHCII (green stain) conveyed Type II muscle fibres. Type I and Type II muscle fibres were divided by cell membrane borders, which were stained with a blue colour by using WGA (wheat germ agglutinin).

2.7.5 Fibre-type Specific CreaT Expression

In this thesis, fibre-type specific immunofluorescence CreaT protein expression was achieved by analysing serial sections combined. For example, as can be seen in **Figure 2.9** sections from the same subject were prepared on two separate slides at the same position. Following the sections management (**Figure 2.9B**). Slide one was incubated with CreaT and dystrophin antibody to determine CreaT protein (captured by ×40 magnification) (**Figure 2.9 A**), and slide two was incubated with MHCI, MHCII and WGA to detect muscle fibre type distribution (captured by × 20 magnification) (**Figure 2.9C**). The sections on slide two were used to identity the muscle fibre-types in slide one. Following this, CreaT expression could be determined in different fibre types.

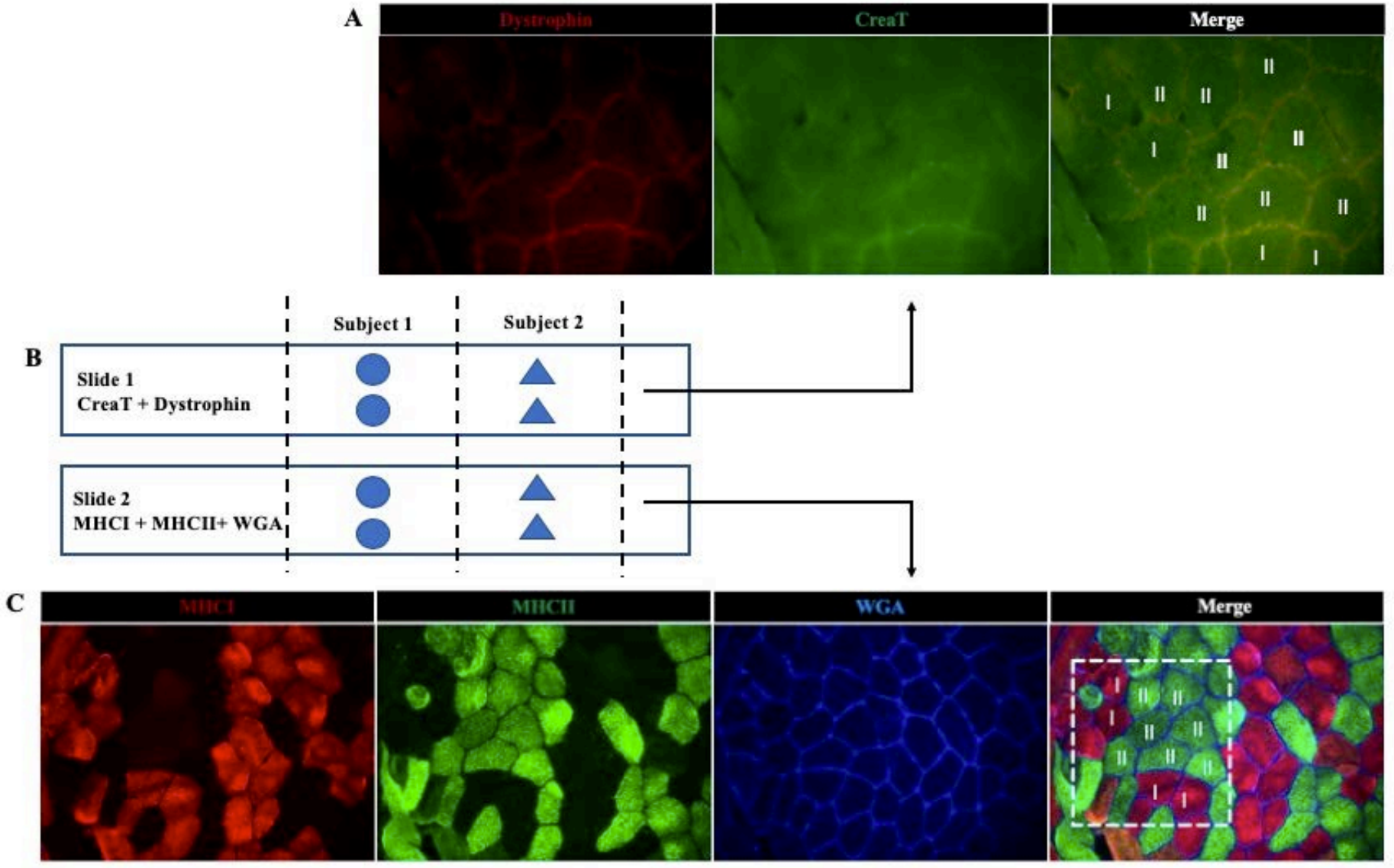

A
Dystrophin
CreaT
Merge
B
Subject 1
Subject 2
Slide 1
CreaT + Dystrophin
Slide 2
MHCI + MHCII+ WGA
C
MHCI
MHCII
WGA
Merge

Figure 2.9 *Immunofluorescent human skeletal muscle fibre type specific CreaT expression.* ***A.*** *Human skeletal muscle marked with CreaT (Green stain) and co-stained with dystrophin (Red stain) to detect CreaT intensity (40X magnification).* ***B****. Sections management to determine fibre specific CreaT expression.* ***C.*** *Human skeletal muscle marked with MHCI (red stain) to detect Type I muscle fibres, MHCII (green stain) to determine Type II muscle fibres and wheat germ agglutinin (WGA) to mark cell membranes. Type I muscle fibres are labelled as I, Type II muscle fibres are labeled as II. Area of (A) are shown in white square of (C)*

2.7.6 CreaT Co-stain with eNOS Secondary Antibody Control

To determine CreaT interaction with the microvasculature, CreaT was co-stained with anti-eNOS antibody, as a marker of capillaries surrounding human skeletal muscle fibres. Secondary antibody control tests were applied to ensure the secondary antibodies were only targeted to either eNOS or CreaT primary antibodies. When CreaT was co-stained with eNOS and both secondary antibodies (**Figure 2.10**A), positive stains of both CreaT (green stain) and eNOS (red stain) were present on images. When eNOS primary antibody was excluded (**Figure 2.10**B), the positive red stain disappeared, indicating the secondary antibody of eNOS does not cross-react with other antibodies used in this experiment. Similarly, exclusion of CreaT primary antibody (**Figure 2.10**C) resulted in a non-positive stain on images, indicating that the secondary antibody of CreaT does not cross-react with other antibodies.

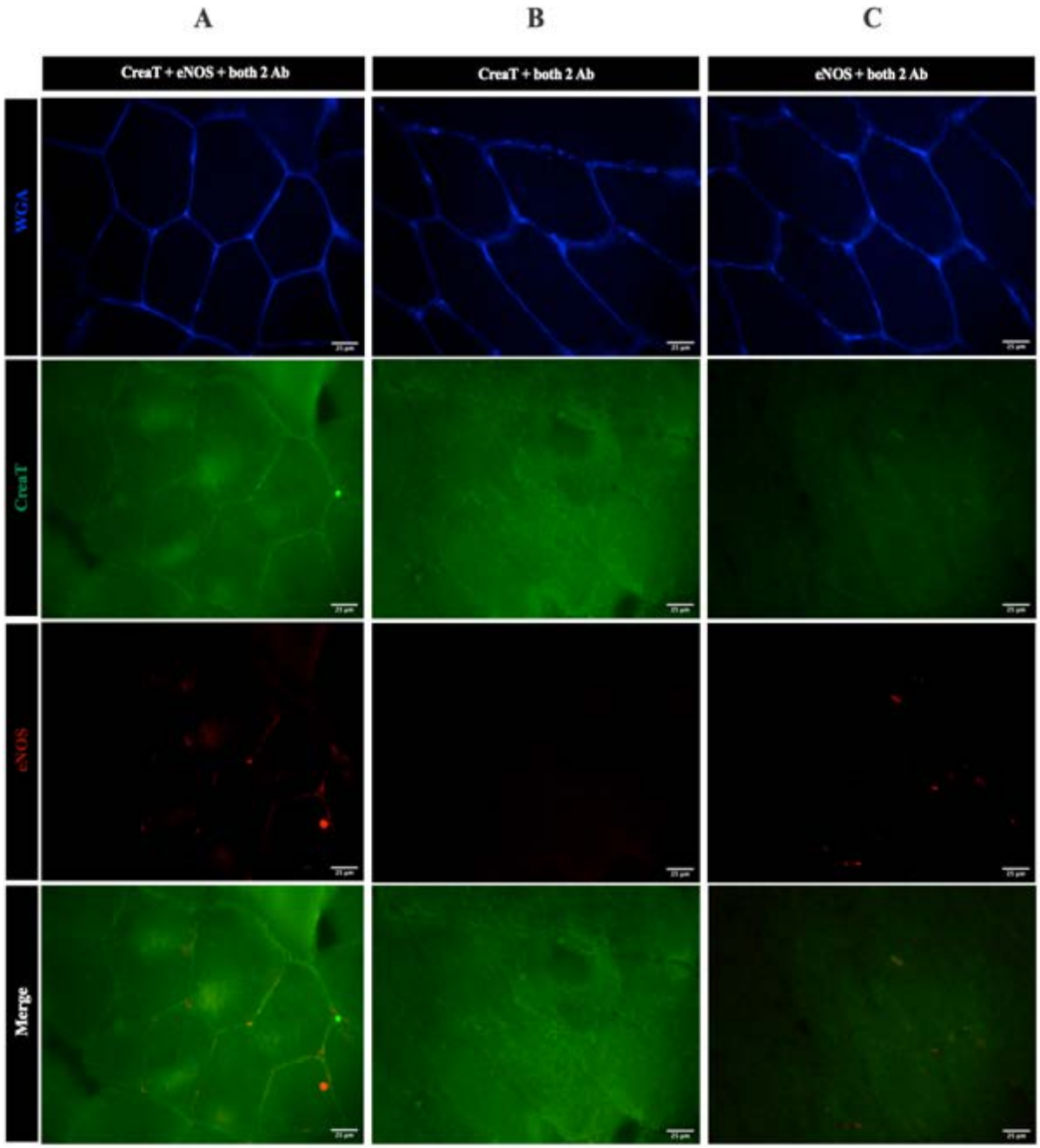


Figure 2.10 *Immunofluorescence detection of CreaT and eNOS antibody control tests. Human skeletal muscle sections were marked with CreaT antibody (Green stain) to determine CreaT expression, endothelial nitric oxide synthase (eNOS) antibody (Red stain) to identify microvascular, Wheat Germ Agglutinin (WGA) was used to indicate cell membranes.* ***A****. Both CreaT and eNOS primary antibody target with their secondary antibodies.* ***B****. CreaT primary*

antibody target with its secondary antibody together with eNOS secondary antibody. ***C****. eNOS primary antibody target with its secondary antibody together with CreaT secondary antibody. All images were captured at the same 40 X magnification. Scale bar on the right bottom of images was set at 25um.*

2.7.7 Creatine Kinase (CK) Antibody Optimization

CK primary antibody was serially diluted at 1:4000 and 1:8000. And the amount of loading protein was tested by 10ug and 20 ug at both dilutions. All secondary antibodies were diluted by 1:10000. The best dilution was determined by the signal strength and relative background noise (**Figure 2.11**). It was determined that a 1:8000 primary antibody dilution loaded at 10ug was the best of the four combinations, due to its lower background noise and the visualised signal.

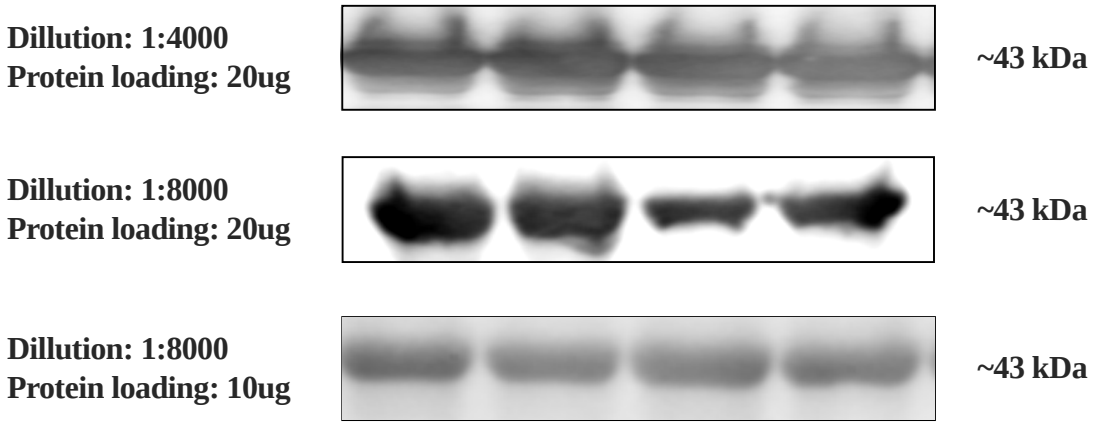


Figure 2.11 *Total Creatine Kinase (CK) antibody dilutions. Three different dilutions were tested in western blot experiments.*

2.8 Attempts Made to Fix with Different Solutions

As fixation plays an important role in the immunofluorescence staining process, comparison between different fixation approaches were tested here (**Figure 2.12**). Acetone and Ethanol (3:1) and 4% Paraformaldehyde (PFA) fixing methods were carried out these experimental tests. There was no visualised difference between the different fixation approaches. However, PFA was chosen for the experiments in this thesis was due to its character in preserving good cellular structure of tissues (10). Furthermore, PFA is a widely used fixation approach and the stability of PFA has been well investigated (11, 13). Therefore, in this thesis, 4% PFA has been used as fixation solution. Two different fix solutions were listed in **Table 2.3.**

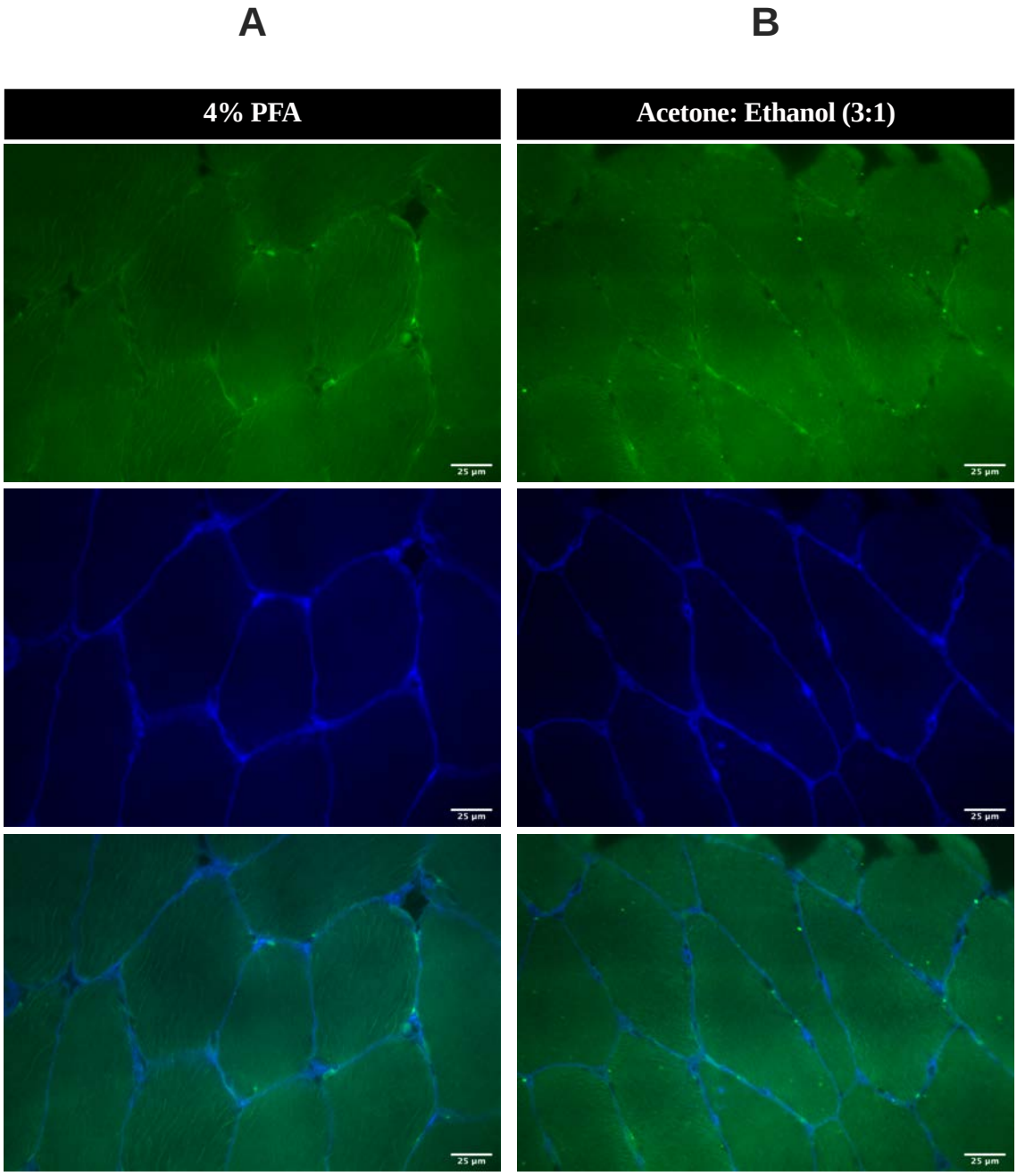


Figure 2.12 *Immunofluorescent stain of CreaT in different fixations. Human skeletal muscle marked with CreaT (green stain) to detect CreaT protein and wheat germ agglutinin (WGA) to mark cell membranes. A. CreaT antibody fixed by 4% paraformaldehyde (PFA); B. CreaT antibody fixed by acetone and ethanol (3:1).*

Table 2.3 *Solutions used for Immunofluorescence staining.*

	Fixation	Permeabilization	Blocking	Mountant
CreaT intensity & Co-localisation	4% PFA	PBST and/or PBS	5% normal goat serum in 1%BSA	Prolong® Gold antifade regent Cat No. P36930
CreaT intensity & Co-localisation	3:1 Acetone to ethanol	PBST and/or PBS	5% normal goat serum in 1%BSA	Prolong® Gold antifade regent Cat No. P36930

2.9 Attempts Made to Co-Stain with MHCI

Previous studies used an immunofluorescence microscopy protocol of CreaT, indicated that Type I muscle fibres have greater CreaT protein compared to Type II muscle fibres (17). However, the Cocktail' antibodies (mixed CreaT and MHC) and negative control (without secondary antibodies) were applies in this study (16, 17). However, it has been reported that CreaT antibodies were experienced some issue in previous study, such as lack of specificity/sensitivity (6, 18, 21), more control tests should be considered in this method development, such as primary antibodies controls (between CreaT and MHCI), lack of critical antibody controls may lead to unreliable conclusions. In the first place, we followed similar immunofluorescence staining protocol of previous study. Significantly difference of CreaT protein distribution was observed between Type I and Type II muscle fibres (show in **Figure 2.13**), however, when we excluded MHCI primary antibody, the greater significant brighter signal disappeared and replaced by a less-bright signal, this results has been shown in **Figure 2.7**B. Therefore, we decided to stain CreaT protein and fibre types individually rather than use a 'cocktail' antibody. A series staining method (described in **Figure 2.9**) was used to determine fibre type specific CreaT distribution.

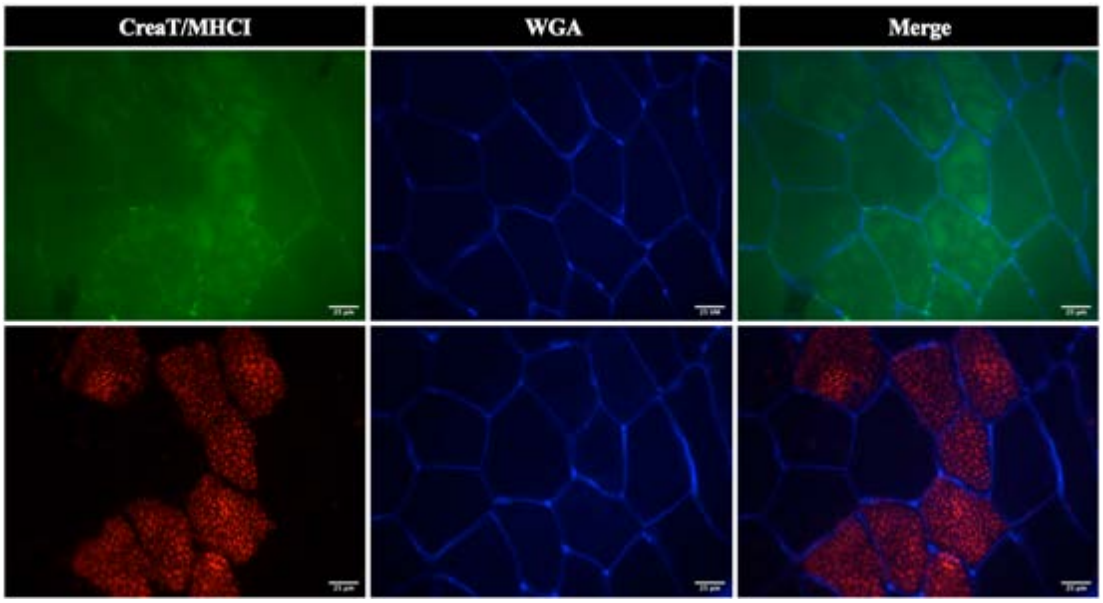


Figure 2.13 *Immunofluorescent CreaT stain with MHCI. Human skeletal muscle marked with CreaT (green stain) to detect CreaT protein and wheat germ agglutinin (WGA) to mark cell membranes. Top panels are CreaT antibody with WGA; bottom panels are MHCI antibody with WGA.*

2.10 Attempts Made to Quantify CreaT Co-Localisation with Dystrophin

CreaT and dystrophin co-localisation are showed in **Figure 2.14**. The co-localisation of CreaT and dystrophin was quantified automatically by the software by using Pearson's correlation coefficient (PCC). The results were occasionally inaccurate due to the indistinct stain of dystrophin (low signal), which may have generated a high PCC despite no detectably greater CreaT signal at cell membrane in images. Therefore, PCC values were not appropriate to determine co-localisation between CreaT and cell borders in this thesis. Instead, an alternative experimental approach to solve the problem was conducted, by analysing a specific region of CreaT protein expression (described above in Section 2.6.8). The reproducibility was quantified as the coefficient of variance (CV), CV value for colocalisation between CreaT and dystrophin was 8.6%.

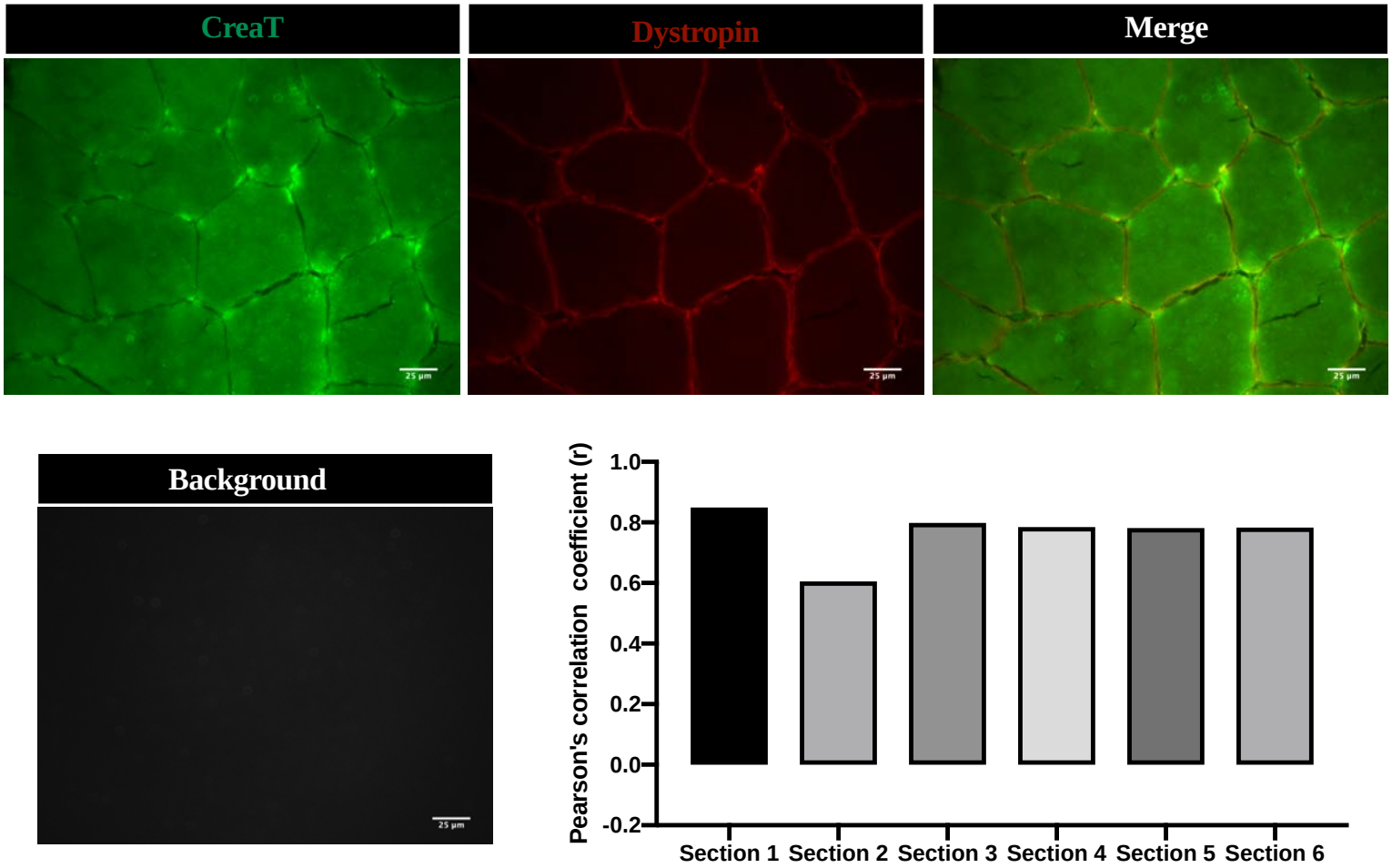


Figure 2.14 *Pearson's correlation coefficient measurement of CreaT and dystrophin co-localisation. Human skeletal muscle marked with CreaT (green stain) to detect CreaT protein and dystrophin to mark cell membranes. All values were eliminated background levels.*

2.11 Discussion

This chapter outlines a validated and optimized method for immunofluorescence and immunoblotting experiments to investigate fibre-type specific CreaT protein content and sub-cellular distribution in human skeletal muscle. Both primary and secondary antibodies were applied to immunofluorescence and immunoblotting methods to successfully target specific proteins of interest in human skeletal muscle, as confirmed via a series of antibody validation control tests to ensure the specificity and avoid potential unspecific binding. An appropriate multiple antibody 'cocktail' co-stain method was optimized to identify fibre-specific CreaT protein content intensity and association with the cell periphery. An appropriate fixation approach was determined and optimized to ensure the quality of immunofluorescence staining. Although, no visualised difference was observed between acetone to ethanol and 4%PFA, 4% PFA was chosen for the experiments in this thesis was due to its character in preserving good cellular structure of tissues.

Immunofluorescence microscopy-derived total CreaT protein was observed in human skeletal muscle in a fibre-type specific manner, with greater expression noted in Type I fibres. This result is in line with previous findings in rodent and human skeletal muscles (4, 16, 17). Although, the present data are consistent with previous studies, following several cross-binding control tests, our experimental results indicated that previous immunofluorescence methods may have potentially resulted in non-specific binding between CreaT protein and MHCI primary antibody, which would erroneously increase the intensity of CreaT protein in Type I fibres. Therefore, extending on the earlier attempts at CreaT staining, our optimized protocol provides a more accurate and reliable indication of fibre-type CreaT protein content and distribution in human skeletal muscle.

The present study demonstrated sub-cellular CreaT protein localisation in specific regions in human skeletal muscle by using a new calculation method (fully described in section 2.6.8). Specifically, we identified that the CreaT isoform with a molecular weight of ~70 kDa was predominantly localized to the cell periphery in Type I and Type II muscle fibres. This finding has been described previously in both rats and human skeletal muscles, but never before quantified as we have done here (19, 21, 25). We initially attempted to use Pearson's correlation coefficient (PCC) to determine co-localisation of CreaT with dystrophin (cell membrane marker) (8). However, this method showed high values of co-localisation (r^2 of ~0.8), potentially due to the low intensity values of dystrophin that generated an insufficient signal in the red channel (8), or due to the high background noise in some sections (8). As PCC values showed variability between different sections, we do not believe this parameter could accurately represent true co-localisation between CreaT and dystrophin. Therefore, we developed a new analysis method to determine CreaT localisation at cell border regions, to confirm that CreaT does indeed reside predominantly at the cell periphery.

Taken together, these validated and optimised methods provide a more precise approach to identify CreaT protein in human skeletal muscle, permitting greater depth of investigation into creatine metabolism in human skeletal muscle. Additionally, these techniques could provide valuable insight into the role of CreaT in alterations in intramuscular Cr metabolism across the spectrum of health and disease. Finally, these methodological advancements provide a platform to further investigate the distribution of CreaT protein and its location-associated functions, (i.e. Cr uptake and energy expenditure) in physiological scenarios of altered muscle morphology and energy status.

Acknowledgement

I would like to thank Dr. Sophie Joanisse, Dr. Andrew Philp and Dr. Leigh Breen for the help and suggestions on developing the immunofluorescences microscopy methods. Dr. Benoit Smeuninx and Dr. Nathan Hodson for their advice on image quantification. Dr. Leigh Breen and Dr. Smeuninx collected the muscle samples for these experiments.

2.12 References

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Chapter 3

Novel Insights into Human Skeletal Muscle Creatine Transporter Expression as a Function of Age

Novel Insights into Human Skeletal Muscle Creatine Transporter Expression as a Function of Age

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ABSTRACT

Background**:* It is well established that the creatine transporter (CreaT) protein plays an important role in intramuscular creatine (Cr) uptake and metabolism. However, the mechanisms of skeletal muscle CreaT regulation remain largely unexplored. Studies have demonstrated that Type I muscle fibres contain greater CreaT protein content, inversely associated with Cr content Additionally, reduced intramuscular Cr and lower CreaT content in a characteristic of some skeletal muscle myopathies and may be implicated in age-related muscle deterioration. ***Purpose: The aim of this study was to used previously validated and optimized immunofluorescent staining and immunoblotting methods to investigate whether CreaT distribution and sub-cellular expression is altered in older age. ***Method:*** A sub-set of quadriceps muscle biopsy samples from 6 young (26.8±4.3 yrs) and older (68.8±2.0 yrs) healthy individuals were used for immunoblotting and immunofluorescence microscopy analysis to identify fibre-type CreaT content and distribution, Creatine kinase (CK) content and high-energy phosphagens. A new developed method of calculation was used to identify CreaT protein intensity in specific sub-cellular regions, referred as ring (sarcolemmal membrane) and centre (non-membrane bound) CreaT intensity. ***Results:*** A CreaT positive stain was visualized in skeletal muscle with no clear age-related difference. CreaT content was greater in Type I compare with Type II fibres in old individuals. Additionally, a greater CreaT content was observed close to sarcolemmal membrane regions (*per* ring area and centre area CreaT content) (Young: 100.69 AU ± 7.18 *vs*. 96.25 AU ± 7.13, P = 0.018, Old: 85.84 AU ± 2.55 vs. 81.82 AU ± 3.02, P < 0.01,) with no fibre-type or age-related differences. Intramuscular CK (Young: 1.33 ± 0.08, Old: 0.98 ± 0.01, P < 0.01) and PCr (Young: 69.33 ± 3.05, Old: 61.09 ± 1.81, P < 0.05) content were lower in old vs. young muscle.

Conclusion: These findings confirmed suggestions that intramuscular creatine metabolism is altered by ageing, and show for the first time that CreaT protein content remains relatively

unaffected. CreaT appears to be mainly distributed close to cell membranes with significant fibre-type specific differences in old but not young individuals, perhaps due to the relatively small sample size used for analysis.

3.1 Introduction

Creatine (Cr) is an non-essential component involved in high-energy phosphate metabolic processes that plays an important role in storage, transport and regulation of cellular energy (54). Cr is primarily synthesized in kidneys and liver, and is transported into muscle cells through the blood stream (58). Nearly 95% of total Cr (TCr) is stored in skeletal muscle as free or phosphocreatine (PCr) to generate high intracellular levels of adenosine triphosphate (ATP) via the creatine kinase (CK) reaction (8, 13). CK is expressed in high-energy demand tissues and organs. For example, CK content is greater in glycolytic Type II muscle fibres compared with more oxidative Type I fibres (34).

Cr supplementation increases intramuscular TCr and the PCr pool (24, 49) which results in a greater ATP buffering capacity, and augments the magnitude of improvement in muscle strength, mass and performance with exercise training (12, 18), albeit with some variation (4, 39). An explanation for the variable response to Cr supplementation may be related to the Cr uptake capacity across muscle cell membranes, which is regulated by creatine transporter (CreaT) proteins (36, 44). Currently, studies of human skeletal muscle CreaT are scarce and, consequently, our understanding of intramuscular Cr transport mechanisms remains poorly defined.

CreaT is currently the only known highly-specific, saturable sodium/chloride (Na^+/Cl^-) gradient-dependent protein (47, 51), thought to be predominantly located at sarcolemmal membranes in human skeletal muscle. Therefore, skeletal muscle CreaT content and sub-cellular distribution is likely to be an important determinant Cr uptake and metabolism. Indeed, muscle myopathies characterised by diminished intramuscular Cr and PCr have been linked to CreaT dysfunction (27, 52, 53). It has also been reported that intracellular Cr content is inversely correlated with the rate of Cr transport (30). Additionally, studies demonstrate that CreaT protein expression is higher in slow-twitch muscles in rodents and humans (11, 36).

Since the last studies of human skeletal muscle CreaT were published >15 years ago, there has been no further exploration of human skeletal muscle CreaT kinetics, using contemporary techniques to understand whether this pathway of muscle metabolic regulation is implicated in pathological conditions of skeletal muscle deterioration, or potentially a suitable therapeutic target.

The aged-related loss of muscle mass and strength, termed sarcopenia (20, 38), is characterized by the reduction in Type II muscle fibre size and number (22, 23). Sarcopenia ultimately reduces physical function, increases risk of frailty, disability and premature mortality (22, 33) and presents a significant socio-economic challenge in the face of global population ageing (20). Given recent evidence that lower-limb intramuscular PCr is reduced in older age (17), Cr supplementation is one strategy with the potential to increase muscle strength/function, cellular bioenergetics and metabolic function in aged skeletal muscle, particularly when combined with exercise training (16, 42). However, although Cr supplementation is generally reported to enhance muscle strength and mass in older individuals, the ergogenic response is inconsistent and/or diminished compared with that seen in younger individuals (17, 19, 39). The potential ergogenic benefits of Cr supplementation in the elderly may therefore be dependent on CreaT content and/or function, although CreaT kinetics as a function of age have yet to be explored (51).

Therefore, the aim of the present study was to confirm i) whether high-energy phosphates for Cr metabolism are altered in ageing and ii) to use newly developed and validated immunofluorescent staining and western blotting methods to determine fibre-type CreaT protein content and sub-cellular distribution in young and old human skeletal muscle. We hypothesized that CreaT would be higher in Type I vs. Type II muscle fibres, and would be located primary at the cell periphery. In addition, we posited that ageing would reduce skeletal muscle CreaT protein content and distribution in a fibre-specific manner, thereby offering a

potential explanation for age-related differences in muscle mass and Cr metabolism reported elsewhere.

3.2 Materials and Methods

3.2.1 Human Skeletal Muscle Collection and Preparation

Ethical approval for the collection of muscle samples was granted by the NHS West Midlands Black Country and Solihull Research Ethics Committees (13/WM/042 and 15/WM/0228) and conformed the principles of the Declaration of Helsinki (7th version). Written informed consent was obtained from all participants before samples were collected. As this study analysis was retrospective, there was insufficient muscle biopsy material available to perform all experiments in tissue the same cohort of young and old individuals. Therefore, samples used for western blot and immunofluorescence microscopy analysis of CreaT and CK were collected from 6 young and 6 healthy older individuals (characteristics presented in Table 2). Samples for enzymatic analysis of intramuscular high-energy phosphates (Cr, PCr and ATP) were determined from biopsy tissue obtained from 8 healthy young and 8 older individuals (characteristics presented in Table 3) in order to confirm earlier reports of diminished PCr in lower limb muscles of older adults (15). All participants were confirmed without any uncontrolled hypertension, diabetic, obese conditions and neuromuscular or cardiovascular diseases. In addition, participants with any medication and smoking history that might affect muscle metabolism were excluded from this study. Muscle biopsies were obtained from the *vastus lateralis* using the Bergstrom percutaneous needle biopsy technique (6), under local anaesthesia. Samples were cleaning by using sterile saline to remove excess blood and dissected free of any visible fat and collagen. Biopsy tissue was then snap frozen in liquid nitrogen (enzymatic analysis and western blot), or placed in Tissue Tek OCT compound and

frozen in liquid nitrogen-cooled isopentane (immunofluorescence microscopy) before being stored at -80℃ freezer until experimental analysis.

3.2.2 Enzymatic Analysis

Approximately 10mg of freeze-dried tissue was powdered, after removal of macroscopic blood and connective tissue, and metabolites extracted in 0.5 M perchloric acid (containing 1 mM EDTA), followed by neutralisation with 2.2M K2CO3 (Sigma Aldrich, Gillingham, UK). Cr, PCr and ATP were determined enzymatically in muscle extracts according to the method of Harris et al, modified to accommodate use of a 96-well plate spectrophotometer. TCr is reported as the sum of PCr and Cr. Metabolite contents were corrected for non-muscle constituents by using muscle ATP content.

3.2.3 Immunofluorescence Staining

The thickness of cryosections was 10μm for all subjects. Details of this experimental procedure are presented in **Chapter 2**.

3.2.3.1 CreaT Intensity

Sections were fixed at room temperature in 4% paraformaldehyde (PFA) solution (Fisher, UK) for 10 min and then washed 3×5 min in phosphate buffered saline (PBS) with 0.2% Tween (PBS-T). Sections were then blocked in 5% normal goat serum (Invitrogen, UK) in 1% bovine serum albumin solution (1% BSA) for 1 h at room temperature and then incubated with anti-SLC6A8 antibody (ab62196, abcam, 1:150, Cambridge, UK) and dystrophin (mouse IgGα, 1:100) mixed antibody in 1×PBS at 4℃ overnight. On following day, sections were washed for 3 × 5 min in 1× PBS-T and secondary antibodies were then applied to sections for 120 min

at room temperature. After secondary antibody incubation, Sections were then left to dry for ~10 min and mounted with 20ul Mowiol ® 4-88 (SIGMA-Aldrich, Gillingham, UK) and covered with glass coverslips. Slides were left to dry overnight in a dark place before capturing images. Primary antibodies, secondary antibody, blocking peptides, experimental dilutions and details of experimental procedures are listed in **Chapter 2**.

3.2.3.2 Muscle Fibre Types

Sections were incubated with Triton X 100 (at 0.02% concentration in 1×PBS) for 5 min and then blocked in 5% normal goat serum (Invitrogen, UK) in 1× PBS for 90 min at room temperature. Sections were incubated with MHCI (IgG2b, BAF8, DSHB, Iowa, US) and MHCII (IgG1, SC.71) mixed primary antibodies (in 1×PBS) at room temperature overnight. The following day, sections were washed for 3 × 5 min in 1×PBS-T and incubated in secondary antibodies and WGA mixed solutions in 1×PBS for 90min at room temperature. Following secondary antibody incubation, sections were then washed 2 × 5 min with PBS-T and 1 × 5 min with PBS and left to dry. The remaining processes was similar with CreaT intensity experiments. Primary antibodies, secondary antibody, experimental dilutions and details of experimental procedures are listed in **Chapter 2.**

3.2.3.3 CreaT Co-localize with Capillaries

Sections were fixed at room temperature in acetone and ethanol (3:1) mixed solution (Fisher, UK) for 5 min and then washed 3×5 min in phosphate buffered saline (PBS) with 0.2% Tween (PBS-T). Sections were then blocked in 5% normal goat serum (Invitrogen, UK) in 1 × PBS for 1 h at room temperature and then incubated with eNOS primary antibody (BD Transduction, #610297, 1:200) and CreaT (ab62196, Abcam, 1:150, Cambridge, UK) mixed antibody in 1×PBS at 4℃ overnight. On the following day, sections were washed 3 × 5 min in 1×PBS-T and incubated in secondary antibodies and Wheat Germ Agglutinin (WGA-350) mixed

solutions in 1×PBS for 60 min at room temperature. The remaining processes were as same as the CreaT intensity experiment. All antibodies and solutions used in this study are listed in Chapter 2.

3.2.4 Image Capture

Wide-field image capture was completed by using Nikon E600 with a 40×0.75 numerical aperture objective. Images per area were captured under three colour filters achieved by a SPOTRT KE colour shot CCD camera (Diagnostic Instruments Inc., MI, USA), illuminated by a 170 W Xenon light source. For image capture, the Texas-Red (540-580mm) excitation filter was used to capture signals of dystrophin (sarcolemma), MHCI (Type I fibres) eNOS (capillaries). FITC (465–495 nm) excitation filter were used to capture signals of CreaT or MHCII (Type II fibres). DAPI UV (340–380 nm) filter was used to view WGA-350 (cell membranes) signals and stains in blue. Wide-field images for CreaT were obtained using 40x objective and muscle fibre types were obtained using 20x objective. All images processing and analysis were carried out in Image Pro Plus 5.1 (Media Cybernetics, MD., USA). CreaT fluorescence intensity was used to identify differences abundance of CreaT protein. Ratio of ring area and centre area mean CreaT protein density was used to indicate the distribution of CreaT around cell membranes.

3.2.5 Immunoblotting

Snap-frozen samples ~30 mg, were homogenised in a buffer (50 mM Tris-HCL, 1mM Ethylene glycol tetra-acetic acid (EGTA), 1 mM Ethylenediaminetetraacetic acid (EDTA), 10 mM B-Glycerophosphate, 50 Mm NaF) with a Protease Inhibitor Tablet (Roche No. 05892791001). Samples were centrifuged at 800xg 10 min at 4°C and the resulting supernatant was aliquot. Protein concentration was determined by a DC protein assay and prepared at equal amounts of

protein in 4xLaemmli sample buffer. Protein (20ug) was loaded and separated on 10% acrylamide gels by SDS-PAGE for 40 min. Protein then transferred to a nitrocellulose membrane (Whatman, Dassel, Germany) at 100V for 1h. Then membranes stained with Ponceau S solution and blocked in 5% skim milk before primary antibody incubation overnight at 4°C. Membranes were wash 3 × 5 min with TBST, and then incubated in goat anti-rabbit IgG Horseradish peroxidase (HRP) conjugated secondary for 1 h at room temperature before washing in TBST and detected by Immobilon Western chemiluminescent HRP substrate (Merck Millipore, Watford, UK). Images were captured by using a G:BOX Chemi XT4 imager with GeneSys capture software (Syngene, Cambridge, U.K.). A Chemi Genius Bioimaging Gel Doc System (Syngene) was used to image and quantify bands. Proteins are expressed relative to Ponceau loading control. Details of antibodies and experimental procedures are listed in **Chapter 2.**

3.2.6 Calculations

Ring area mean integrate density was used to identify CreaT protein content intensity around cell membranes. CreaT intensity and density are present by using a relative unit of measurement to show the ratio of amount of substance and refer as arbitrary unit (a.u. or AU). Details of calculation are listed in Chapter 2.

3.2.7 Statistics

SPSS 22 was used for all statistical analyses. An unpaired student T-test was used to compare i) muscle high phosphagens, ii) fibre CSA and fibre proportion iii) total intramuscular CreaT content, iv) creatine kinase, v) TCreaT intensity and vi) muscle fibre-type specific CreaT intensity between young and old. A repeated measurement was used to compare CreaT protein content in *per* CSA and CreaT protein content in ring and centre area. Significance was set at

$P < 0.05$. Data are reported as Mean ± SD.

3.3 Results

3.3.1 Participant Characteristics

There were no significant differences in body mass, height, BMI, fat mass, fat-free mass, or appendicular lean body (ALM) mass which involved in analysis of intramuscular CreaT and CK protein content (**Table 3.1**). No significant differences were observed in body mass, height or BMI between young and old participants included in analysis of intramuscular high-energy phosphates (**Table 3.2**).

Table 3.1 *Participant characteristics and intramuscular CreaT and CK protein content.*

	Young (n=6)	Old (n=6)	P value
Male/Female	3/3	4/2	-
Age (yrs)	26.8 ± 4.3	68.8 ± 2.0**	0.001
Body Mass (kg)	71.9 ± 14.0	78.1 ± 17.6	0.69
Height (m)	1.8 ± 0.1	1.7 ± 0.1	0.40
BMI (kg/m^2)	23.2 ± 2.9	26.9 ± 5.5	0.19
FM (kg)	18.9 ± 6.1	24.2 ± 11.5	0.34
FFM (g)	49.2 ± 10.6	50.1 ± 8.9	0.81
% FFM	68.5 ± 6.3	65.0 ± 7.7	0.41
% FM	26.5 ± 6.9	30.4 ± 8.5	0.40
ALM (kg)	29.9 ± 14.0	22.6 ± 4.8	0.13

*BMI: bod mass index, FM: fat mass, FFM: fat-free mass, ALM: appendicular lean mass. Significant difference was set at $P < 0.05$, indicates a significant difference from young, ** represent significant difference $P < 0.01$. Values are present as mean ± SD.*

3.3.2 Intramuscular High-energy Phosphates

There was no significant difference observed in ATP, free Cr and TCR (**Table 3.2**). PCr was significantly lower in old compared with young individuals ($P < 0.05$).

Table 3.2 *Participant characteristics and intramuscular high-energy phosphates.*

	Young (n=8)	Old (n=8)	P value
Male/Female	5/3	6/2	-
Age (yrs)	22.1 ± 2.9	71.1 ± 3.9**	<0.001
Body Mass (kg)	79.3 ± 5.2	79.1 ± 7.4	0.95
Height (m)	1.8 ± 0.1	1.8 ± 0.1	0.84
BMI (kg/m^2)	24.6 ± 2.0	24.7 ± 2.2	0.92
ATP (mmol/kg/dry wt)	27.39 ± 3.33	24.00± 3.91	0.08
Free Cr (mmol/kg/dry wt)	52.14 ± 4.06	55.58± 4.58	0.13
PCr (mmol/kg/dry wt)	69.33 ± 6.95	61.09 ± 5.11*	0.02
TCr (mmol/kg/dry wt)	121.46 ± 7.62	116.66± 7.05	0.99

*BMI: bod mass index, ATP: adenosine triphosphate, Cr: creatine, PCr; phosphocreatine, TCr: total creatine. Significant difference set at $P < 0.05$, indicates a significant difference from young * represent significant difference $P < 0.05$, ** represent significant difference $P < 0.01$. Values are present as mean ± SD.*

3.3.3 Intramuscular CreaT and CK Protein

There was no significant difference in western blot-derived CreaT protein content between young and old muscle (**Figure 3.1A**) ($P = 0.166$). CK protein content was significantly higher in young compared with old muscle ($P < 0.01$, **Figure 3.1B**).

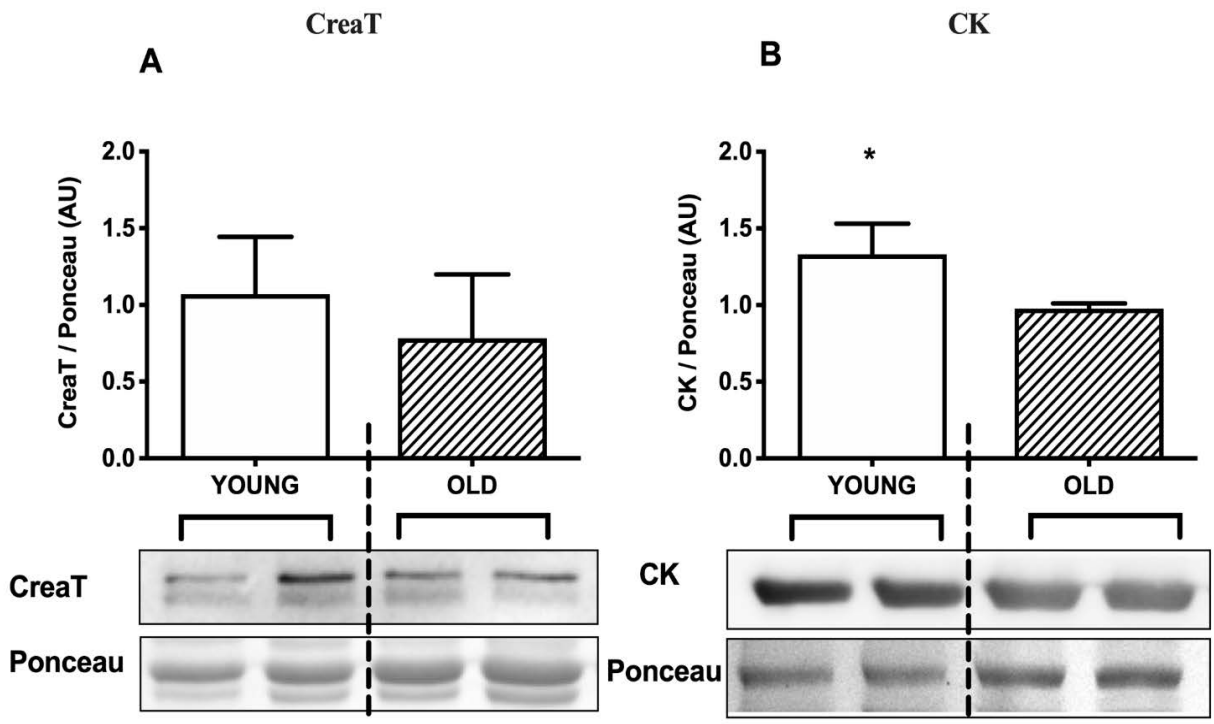


Figure 3.1 *Intramuscular total CreaT protein (A) and total CK (B).Values are presented as mean ± SD. Significant difference was set at P < 0.05. * represent P<0.05. Comparison between groups are tested by Un-paired test.*

3.3.4 Skeletal Muscle Fibre Type CSA and Composition

Chapter 4 Immunofluorescence microscopy of skeletal muscle Type I and Type II fibres were stained on both young and old skeletal muscle tissues (**Figure 3.2 A & B**) The proportion of type II muscle fibre is significant greater compare to type I muscle fibres in young individuals, no significant difference of fibre type proportion was observed in old muscles ($P < 0.05$) (**Figure 3.2D**). No significant differences of type I and type II cross section area (CSA) were observed between young and old individuals (**Figure 3.2C**).

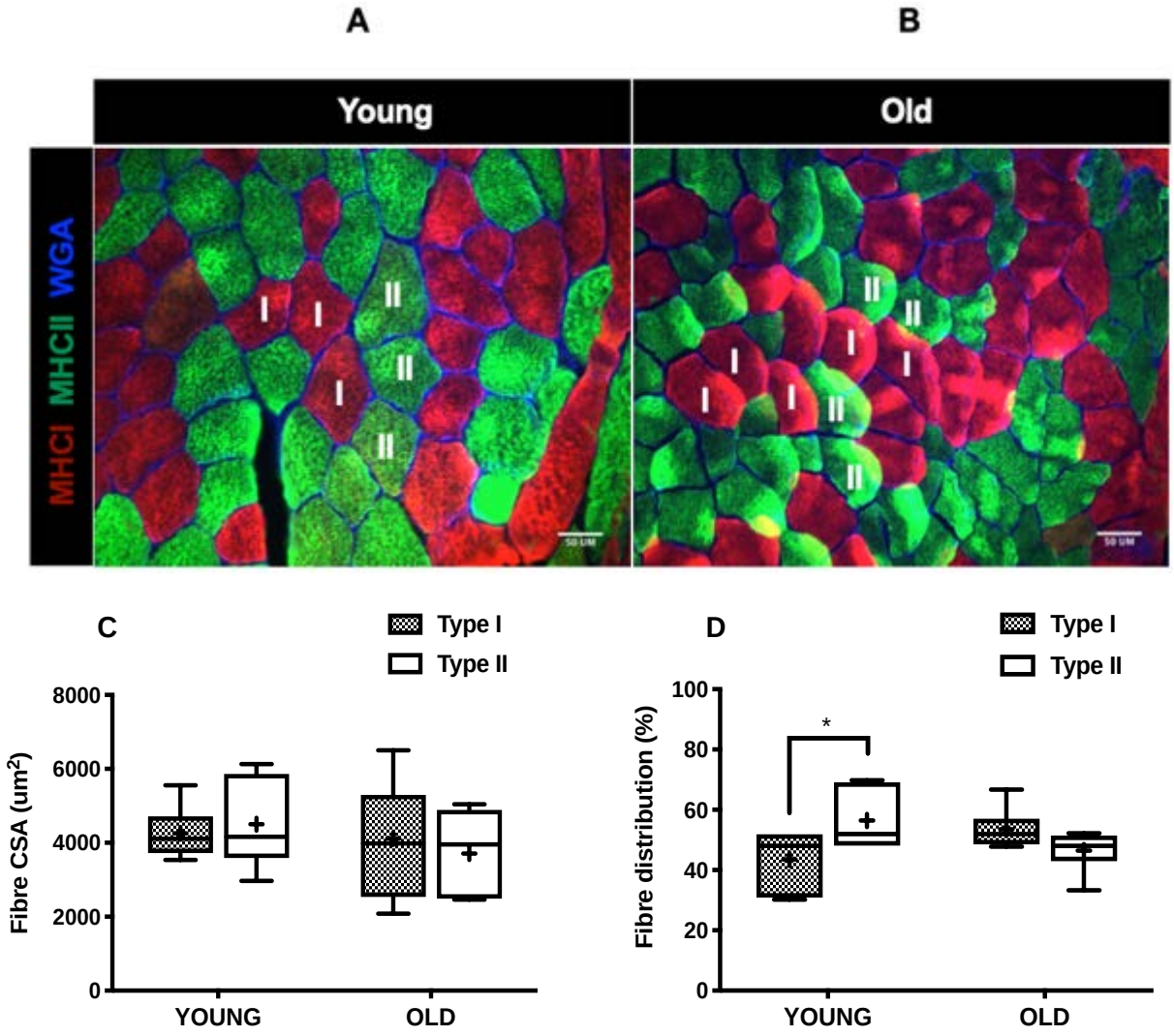


Figure 3.2 *Human skeletal muscle fibre distribution (A) and cross section area (CSA) (B). Sections marked with MHCI (Red stain), MHCII (Green stain) and WGA (Blue stain) in Young (a) and Old (b) muscles. Box whisker plots representing median, interquartile range (IQR), and minimum and maximum values of Type I and Type II muscle fibre type distribution (A) and CSA (B) values in 6 young and 6 old subjects. + shows mean values, significant levels were set at P<0.05. * represent significant difference P < 0.05.*

3.3.5 CreaT Protein Content

There was no significant difference in CreaT protein intensity between young and older

individuals (**Figure 3.3**).

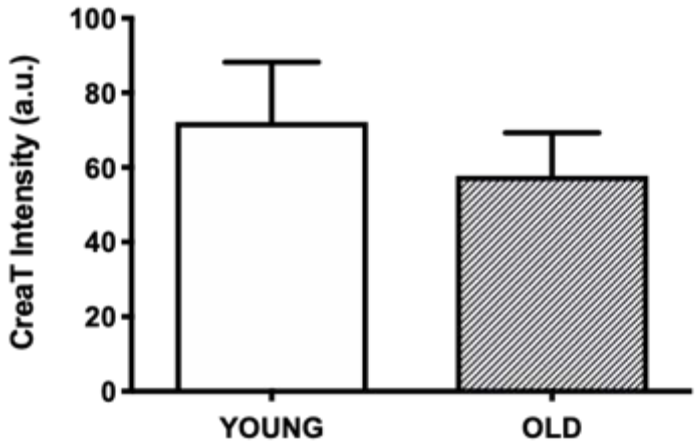
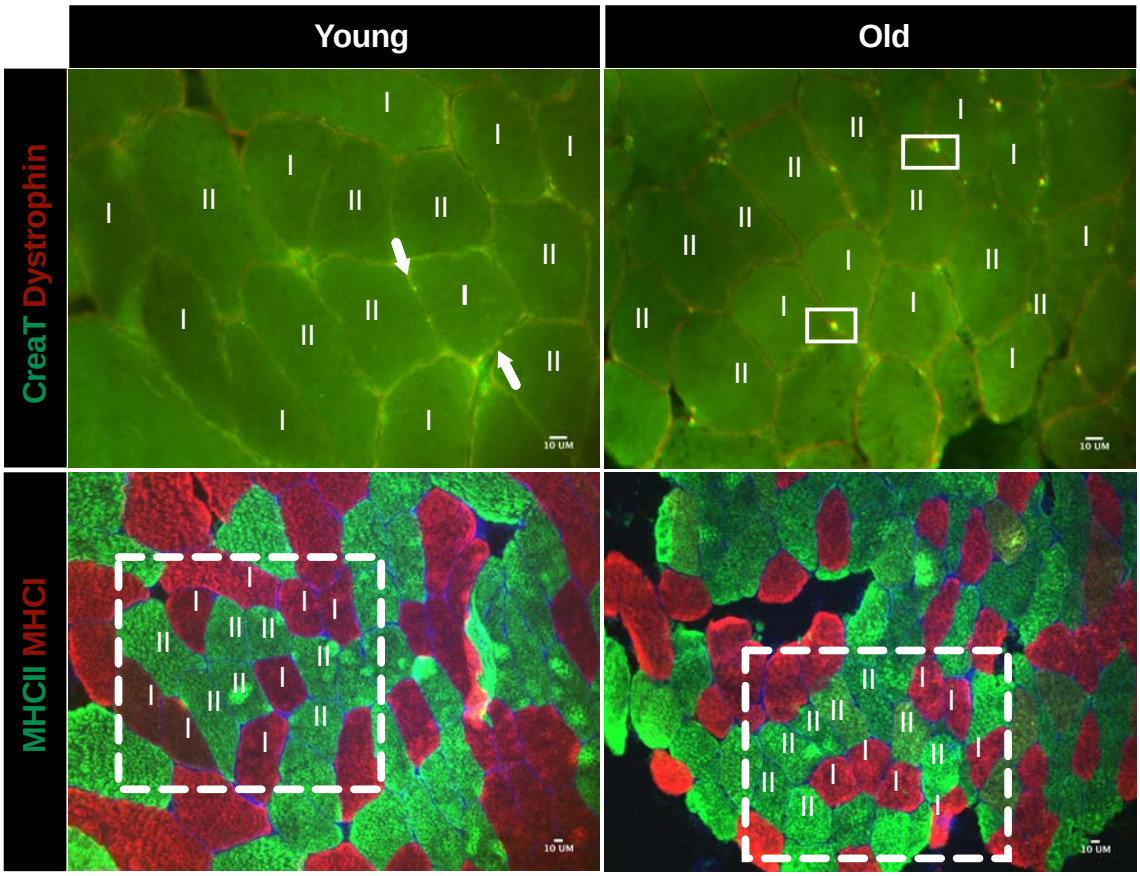


Figure 3.3 *CreaT protein intensity in young and old skeletal muscle tissues. Values are Mean ± SD. Repeated measurements were used to analysis the difference between young and old individuals. Significance was set at P < 0.05.*

3.3.6 Fibre-type CreaT Protein Content

Immunofluorescence microscopy of fibre-type CreaT content is presented in **Figure 3.4A.** There was no significant difference in CreaT protein intensity between Type I and Type II muscle fibres in healthy young individuals (Type I: 95.82 ± 25.64 vs. Type II: 97.57 ± 23.87, P = 0.73). However, in older healthy adults, CreaT protein intensity was significantly greater in Type I compared with Type II muscle fibre (Type I: 84.01 ± 10.59 *vs.* Type II: 80.53 ± 9.99, P = 0.004) **(Figure 3.4B).**

A


Young
Old
CreaT Dystrophin
MHCII MHCI
10 UM

B

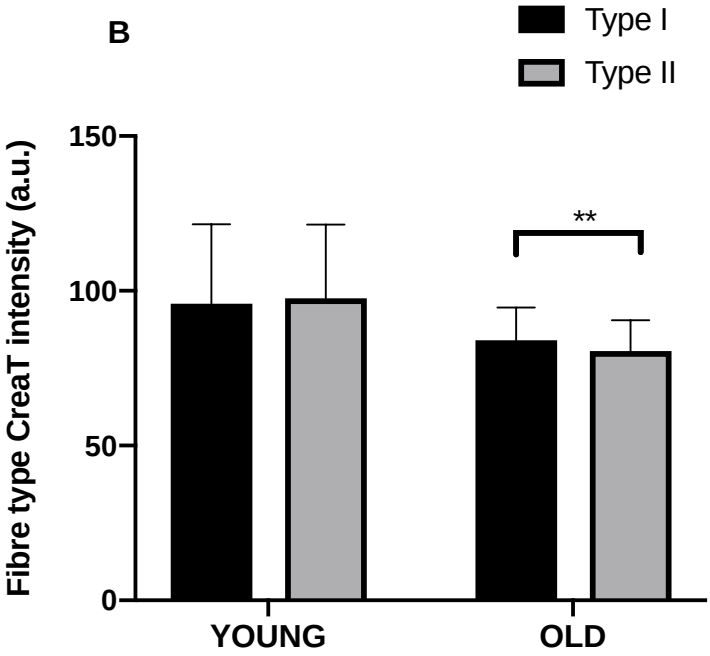

Type I
Type II
Fibre type CreaT intensity (a.u.)
150
100
50
0
**
YOUNG
OLD

Figure 3.4 *Serial sections detections of fibre type specific CreaT protein intensity.* ***A.*** *Top panel is Immunofluorescent (40X magnification) detection of CreaT protein content and localisation. Sections marked with CreaT (Green stain) and co-stained with dystrophin (Red stain). The small white box of top panel shows CreaT distribute at cell membranes as the bright spots cluster on old skeletal muscle samples. Bottom panel is Immunofluorescent (20X magnification) detection of Type I and Type II muscle fibres in young and old individuals. Sections marked with MHIC (Red stain) and MHCII (Green stain). Type I muscle fibres are labelled as I, Type II muscle fibres are labelled as II. Areas of top panel are shown in white square of bottom panel. Arrows in A indicate the bright cell membranes.* ***B****. CreaT protein intensity in Type I and Type II muscle fibres. Values are Mean ± SD. Repeated measurements were used to analysis the difference between young and old individuals. Significance was set at P < 0.05. * shows significant difference P < 0.05, ** shows significant difference P < 0.01.*

3.3.7 CreaT Protein Sub-cellular Location

CreaT protein mean intensity in the ring area was significantly greater than centre area in both young and older individuals (Young: Ring area 100.69 ± 23.73 vs. Centre area 96.25 ± 23.56, P = 0.02; Old: 85.84 ± 8.54 vs. 81.82 ± 10.13, P < 0.01, **Figure 3.5A**). No significant difference of specific areas (ring and centre) CreaT protein intensity between young and older participants. However, while normalised into fibre Type In combination with specific areas, centre area Type I muscle fibre CreaT intensity showed significant difference compare to Type II fibre in old individuals (Centre, Type I: 83.57 ± 4.08 vs. Type II: 80.07 ± 3.82, P <0.01, **Figure 3.5C**). In addition, ring area CreaT intensity in Type I muscle tended to be significantly greater than Type II muscle fibres (Ring, Type I: 87.15 ± 4.08 vs. Type II: 84.53 ± 3.82, P = 0.054, **Figure 3.5B**). No significant differences of CreaT content were observed in young individuals in fibre type specific ring and centre areas.

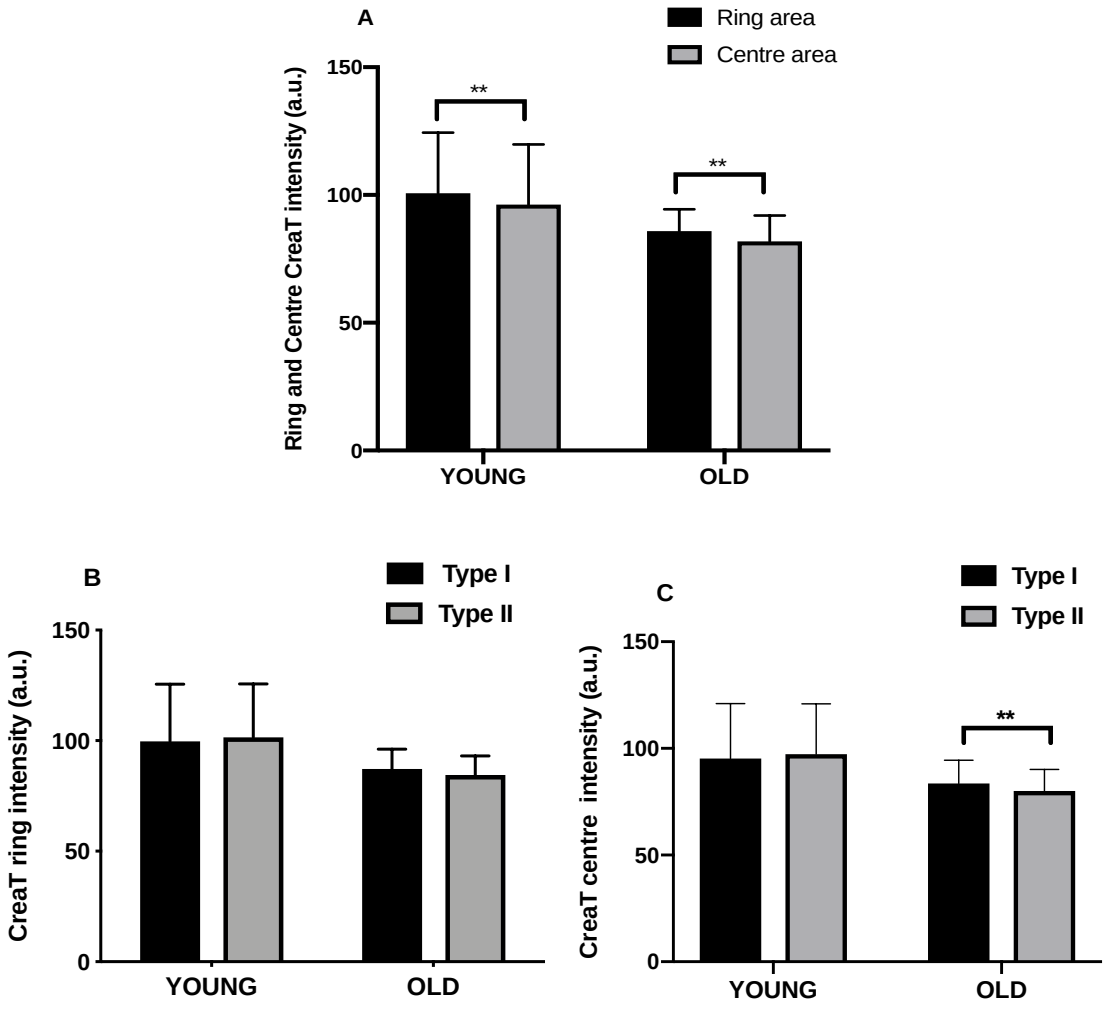


Figure 3.5 *Intramuscular CreaT protein intensity in ring and centre area.* ***A****. CreaT intensity in ring and centre areas.* ***B****. Ring area fibre type specific CreaT intensity between young and old.* ***C****. Centre area fibre type specific CreaT intensity between young and old. Values are Mean ± SD. Repeated measurements were used to analysis the difference between young and old individuals. Significance was set at P < 0.05. * shows significant difference P < 0.05, ** shows significant difference P < 0.01. Ring areas are the areas that selected 10 pixel units' distance from cell membranes towards to centre of cells.*

3.3.8 Intramuscular CreaT and Capillary Co-localisation

Immunofluorescence microscopy results showed that capillaries were localized at cell membranes **Figure 3.6**. However, due to the quantification methods used, we are unable to accurately estimate co-localisation between eNOS (used to detect capillaries) and CreaT. Visually, no apparent difference of eNOS co-localized together with CreaT protein was observed between young and older individuals.

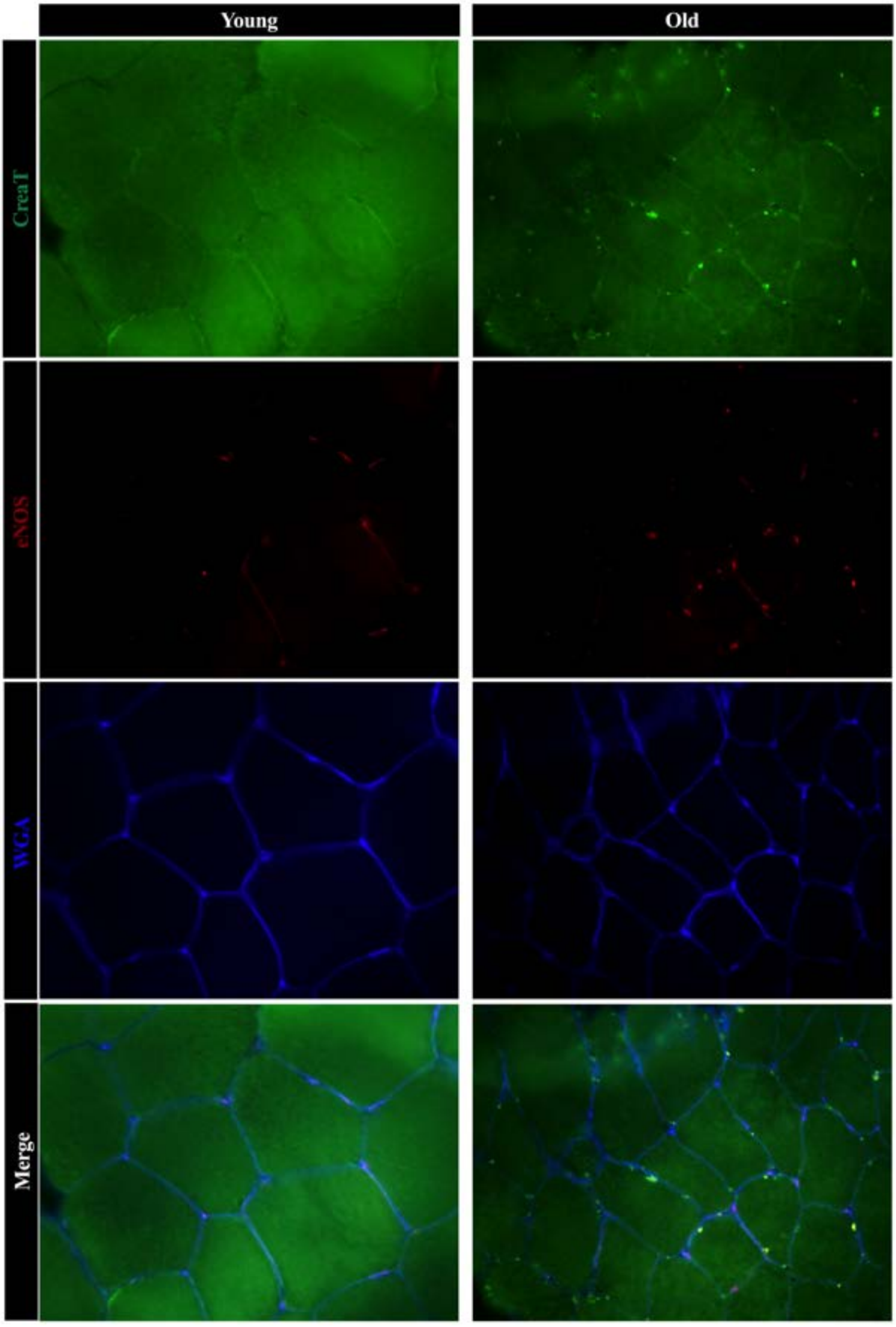

Young
Old
CreaT
eNOS
WGA
Merge

Figure 3.6 *Immunofluorescent detection of CreaT and eNOS. Sections were marked with CreaT (Green stain) and co-stained with endothelial nitric oxide synthase (eNOS) (Red stain) to identify capillilaries in skeletal muscle. Wheat Germ Agglutinin (WGA) was used to mark cell membranes. Images from two individuals in young and old group are displayed. All images were captured at the same magnification (40X).*

3.4 Discussion

The present study findings confirmed previous reports that lower-limb intramuscular phosphocreatine (PCr) may be reduced in older vs. younger individuals, which may explain the well described decreased in exercise performance fatigue in aged skeletal muscle (9, 30, 34). Further to these data, in a separate sub-set of muscle biopsy samples, we demonstrated no discernible difference in muscle fibre-specific CreaT protein content or sub-cellular localisation between young and older individuals. However, immunoblotting revealed that creatine kinase (CK) content was greater in young compared with older muscle tissues.

These findings are in line with previous research which showed the PCr content is lower in older vs. young healthy skeletal muscle (5, 35). Age-related muscle loss is associated with a reduction in muscle fibre size and number, particularly in Type II fibres (3, 45, 50). However, in our study, we did not find any significant difference in fat-free mass, fibre number or cross section area (CSA) between young and older individuals, potentially due to the healthy status of our older volunteers, and limited sample size. Ultimately, the absence of any clear phenotypic or muscle morphological difference between young and older individuals may explain why we did not detect any difference in CreaT protein content between young and older skeletal muscle. Nonetheless, our data clearly indicate that. Analogous to our findings, previous research reported that CreaT mRNA is not altered by ageing (55) and increased intracellular Cr levels through Cr supplementation occurred in the absence of any change in

CreaT content (49). Therefore, it is possible that CreaT protein content may be relatively stable across the adult life-course in healthy muscle cells as chronological ageing *per se* does not appear to alter intramuscular CreaT content or sub-cellular localisation. The activity of CreaT is closely associated with two electrochemical gradients in Na^+ and Cl^- and it has been reported that Cr requires 2 Na^+ ions to provide the energy for entry into muscle cells (10). An insufficient concentration of ions could potentially result in a lower Cr uptake rates via CreaT protein (1) and may explain the lower PCr in ageing reported here and elsewhere (17), but unchanged CreaT content. In line with this notion, it has previously been shown that replacement of NaCl gradient with a potassium or lithium chloride gradient in an extravehicular medium, completely abolished Cr transport (61). Nevertheless, studies reported that CreaT enters muscle cells only need a small portion of ions (11). Therefore, any reduction in ion concentration may fully explain the potentially blunted capacity of creatine transport into muscle cells in ageing.

Skeletal muscle is incapable of Cr synthesis and the intracellular Cr pool is dependent on the rate of Cr uptake. Therefore, the concentration of intramuscular Cr may be an important determinant of CreaT protein regulation. It has been determined that the transport of Cr through CreaT is a saturable process (58). Indeed, an increase in Cr uptake rate is related to a decrease in intramuscular Cr concentration (39, 48). This has been supported by Cr supplementation in vegetarians, who display low levels of intramuscular Cr. However, following 5 days of supplemental Cr loading, the Cr pool increased robustly in vegetarians to a similar level in omnivores. These findings suggest that a greater Cr uptake rate with Cr supplementation may be due to greater CreaT content and/or transport capacity (62). In present study, we did not observe a significant difference of TCr between young and older individuals. It is possible that the similar TCr content between groups would be accompanied by similar Cr uptake rate, which may explain why we did not observe any difference in CreaT content between young and older muscles. Finally, as PCr is an energy shuttle in ATP re-synthesis (52, 58), a decreased

PCr reported in aged muscle may alter energy regeneration processes and stimulate the activation of energy related signalling pathways, such as AMPK (7, 31). Although speculative, the changes of AMPK activity in aged skeletal muscle may explain the corresponding alterations in muscle PCr despite similar CreaT content. Previous studies determined that ageing is associated with the reduction of AMPK signalling capacity and therefore interrupting cellular homeostasis (48). AMPK activity was reported to play roles in muscle metabolic adaptation through the oxidative capacity of mitochondrial (30). Considering the important function of PCr/Cr is reflected as an energy carrier to shuttle high-energy phosphagens between mitochondrial and cellular ATP consumption site (20). Furthermore, PCr/Cr also plays a role in preventing excessive levels of ADP in cells and controls the ratio of ADP to ATP (34), which is important for the production of oxidative energy in mitochondria. Therefore, AMPK activity may alter muscle PCr in aged skeletal muscle.

In the present study, staining of CreaT in human skeletal muscle tissue displayed a strong immuno-reactivity at the cell membrane, which was confirmed by the higher ring area CreaT protein intensity levels around sarcolemma membrane compared to centre area CreaT content in young and older adults. These findings are aligned to earlier work, in which CreaT was reported to localize predominantly at the cell periphery in Type I and Type II muscle fibres (41, 55, 63). One unanticipated finding was intramuscular CreaT protein content did not differ between Type I and Type II muscle fibres in young individuals, despite being significantly greater in Type I vs. Type II fibres in older individuals. The absence of fibre-type difference in CreaT in younger individuals is in contrast with the findings of Murphy et al. (38). The conflicting findings in fibre-specific CreaT content between young and old muscle may due to the small sample size and higher variance in CreaT intensity observed, whereas older muscle CreaT intensity in this study was highly consistent across individuals. The predominant localisation of CreaT at the cell membrane is aligned to its role in Cr uptake and restoration

after scenarios of cellular energy depletion, such as high intensity exercise (58). The peripheral localisation of CreaT also corresponds to its purported role as a neurotransmitters (22, 44, 47). It stands to reason that a stable expression of CreaT at cell membranes is important to maintain intermuscular Cr level and energy homeostasis.

Skeletal muscle is perfused by a complex network of capillaries and other micro-vessels, that provide working tissues with a supply of oxygen and nutrients. As Cr transport into muscle cells occurs via the bloodstream, we also considered the role of skeletal muscle capilliarization in transporting Cr and determined co-localisation between CreaT and these blood vessels. Muscle eNOS protein content has been shown to be correlated with capillary density (27) and predominantly localizes near sites of mitochondrial respiration (27, 54). In the present study, due to the analysis technique used, we could not accurately quantify the co-localisation of CreaT and eNOS. In any case, visual inspection of images did not reveal a strong immuno-reactivity between CreaT and eNOS. Irrespective, it would be prudent to better understand the relationship between CreaT and eNOS in older age. Of particular interest, would be the study of CreaT and eNOS kinetics and complex interactions in response to exercise and Cr supplementation, as a possible explanation to some of the observed variability in the anabolic response of aged muscles to these non-pharmacological stimuli.

There are some limitations in this study. Firstly, as a small-scale pilot investigation, this study included just six young and six older individuals for analysis. Thus, with a small sample size, caution must be applied to our findings, which may not best represent the wider young and older populations. Additionally, this study included both male and female participants. Gender difference may also be a considerable factor that influences intramuscular Cr regeneration, as females generally present lower CK levels and tissue enzyme activity (2, 14). In addition, males are shown to have greater response to Cr supplementation in fat free mass compare with females (14). Intramuscular Cr concentration is highly correlated with skeletal muscle mass,

the lower muscle strength and smaller muscle mass in female (28) potentially vary the TCr levels. Further research of CreaT protein expression and Cr metabolism should consider gender-related differences in age-related studies of CreaT kinetics.

3.5 Conclusion

In conclusion, this study has applied the immunofluorescence method that developed in chapter 2 to visualise the content and localisation of CreaT in young and older individuals. The present findings suggest that aspects of intramuscular creatine metabolism are altered by ageing, whereas CreaT protein content remains relatively unaffected, and may potentially be stable across the life-course in healthy individuals. CreaT protein mainly distributes around cell membranes in young and older individuals, and demonstrated a clear fibre-type difference in old individuals, that was not apparent in the young. These findings provide a platform to investigate whether fibre-specific CreaT expression/activity is a rate-limiting step in the muscle anabolic response to exercise and supplemental creatine interventions that may support muscle mass and strength with advancing age. Future studies should now focus on further elucidating the possible underlying mechanisms of altered cellular energy status in older age. Additionally, identifying how other physiological scenarios of altered muscle morphology and cellular energy status, such as disuse and Cr supplementation, regulate CreaT content and expression should also be undertaken. As CreaT is the essential mechanism by which Cr enters muscle cells, approaches to maintain intramuscular CreaT protein content or activity could have substantial relevance in the prevention or treatment of conditions of musculoskeletal deterioration.

Disclosures

The authors have no conflicts of interest to declare.

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Authorship statement

All authors gave their final approval of the version of the article to be published. DL, SJ and LB designed the study. BS and LB organized and carried out the clinical experiments with the assistance of DL. DL, SJ and LB performed all data analyses. DL, SJ and LB performed the statistical analysis of the data. DL and LB wrote the manuscript together. DL and LB are the guarantors of this work and take responsibility for the integrity and accuracy of the data analysis.

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Chapter 4

Human Skeletal Muscle Creatine Transporter is Altered by Disuse-induced Muscle Deterioration in Healthy Young Individuals

Human Skeletal Muscle Creatine Transporter is Altered by Disuse-induced Muscle Deterioration in Healthy Young Individuals

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ABSTRACT

Background: Periods of physical disuse (inactivity/immobilization0) results in the loss of muscle mass and strength, through alterations in protein turnover that may be related to dysregulated energy homeostasis. Specifically, postabsorptive and postprandial muscle protein synthesis rates are down-regulated with disuse alongside a reduction in the content of high energy phosphates content in muscle. However, the driven mechanism of these alterations is unknown, but may relate to alterations may relate to Creatine transporter (CreaT) expression which is essential for Cr entry into muscle cells. ***Purpose:*** The aim of this study was to investigate the effect of immobilization on CreaT protein expression and distribution in human skeletal muscle. ***Method:*** An optimized immunofluorescence microscopy technique was applied to recognize CreaT in human skeletal muscle. Following this, skeletal muscle samples from 15 healthy young individuals (23.1 ± 3.5 yrs) were used analyzed for CreaT distribution and expression in different fibre types after 7-days unilateral leg immobilization (IMMOB) compared with a non-immobilized control limb (CTRL). A new calculation method was developed to identify CreaT protein intensity in specific muscle fibre regions, referred to as ring (cell membrane) and centre (non-membrane bound) CreaT intensity. ***Results:*** Skeletal muscle isometric strength was significantly lower in IMMOB vs. CTRL after 7-days immobilization ($P < 0.01$), along with significantly lower leg fat free mass ($P < 0.01$). Skeletal muscle Type II fibre cross-section area (CSA) was significantly smaller in IMMOB compared with CTRL ($P = 0.028$). No significantly difference of Type I fibre CSA was observed between IMMOB and CTRL ($P = 0.07$). CreaT mean intensity was greater in IMMOB vs. CTRL ($P < 0.01$), with significantly greater expression in Type I fibres ($P < 0.01$). Within each fibre-type, ring area CreaT mean intensity was greater than centre area intensity in both IMMOB and CTRL ($P < 0.01$). The greater CreaT content in IMMOB vs. CTRL may be partly explained by

a trend for greater CreaT in Type I fibres (P = 0.074). ***Conclusion:*** In summary, we report higher CreaT protein expression throughout muscle fibres following short-term limb immobilization. The greater muscle fibre CreaT expression with immobilization may have important implications for skeletal muscle energy production during disuse-induced atrophy and in the recovery of muscle mass upon re-loading.

4.1 Introduction

Short periods of disuse result in a considerable loss of skeletal muscle mass (8, 36). The loss of muscle mass with repeated episodes of disuse over the life-course may ultimately lead to impairments in strength, functional capacity and increased risk of disability and metabolic disease (35). The reduction of skeletal muscle mass and strength during disuse events is generally accompanied by a lengthy recovery time, a higher risk of injury and impaired metabolism (19, 33). Therefore, given the important clinical implications of short-term disuse, it is imperative to understand the driving mechanisms in order to develop effective strategies to attenuate disuse-induced muscle deterioration. Previous studies suggest that disuse-induced muscle atrophy results from alterations in muscle protein turnover that could, potentially, be linked to dysregulated energy homeostasis (34, 35). Specifically, muscle protein synthesis and breakdown may be adversely affected in the first few days of disuse, during which the rate of muscle atrophy is most rapid and muscle attempts to restore or rebalance cellular energy homeostasis (35).

The phosphagen system uses phosphocreatine (PCr) to generate and maintain ATP at a very rapid rate (2). The only known source of Cr entry into skeletal muscle cells is through the creatine transporter (CreaT) (9). It has been demonstrated that disuse-induced muscle atrophy is accompanied by a reduction in intramuscular high-energy phosphagens (16, 21), which could be an important mediator of the muscle atrophy process. However, the mechanistic link between alterations in muscle mass and high-energy phosphagens during disuse are yet to be investigated, but may be related to alterations in CreaT content and sub-cellular localisation, as an important gate-keeper for Cr entry into cells and mediator of intracellular PCr and/or Cr concentrations. Accordingly, the aim of this study was to use newly developed immunoflouresence microscopy techniques to determine the effect of immobilization on CreaT protein expression and sub-

cellular distribution in human skeletal muscle. We hypothesized that, alongside expected reductions in muscle mass and strength, CreaT protein expression and cellular distribution would be lower with immobilization, aligned to observations from others that disuse diminishes intramuscular PCr.

4.2 Methods

4.2.1 Ethical Approval

Human skeletal muscle samples were obtained from School of Sport, Exercise and Rehabilitation Sciences, University of Birmingham. Ethical approval for the collection of muscle samples was granted by the NHS East Midlands Research Ethics Committees (17/EM/0086) and conformed the principles of the Declaration of Helsinki (7^{th} version). Written informed consent was obtained from all participants before samples were collected.

4.2.2 Participants

Fifteen recreational healthy young male participants aged between 18-35 years old were recruited to complete 7-days of unilateral leg immobilization, with no history of regularly trained resistance or endurance training (in the past 3 years duration). All participants were confirmed without any diabetic, obese conditions and had no-smoking history. The purpose of the study was clearly explained to the participants and a general health questionnaire was assigned before recruitment to identify any potential known cardiovascular or metabolic disorder that would endanger them during testing. All participants were informed of the nature and the possible risk of this experimental procedure before they sign the consent of take part in this study.

4.2.3 Experimental Design

Participants firstly performed an assessment of leg strength on a customised machine to determine the isometric strength. Following three days activity, participants was reported to lab to undergo assessments of height, weight and a 7-min dual-energy x-ray absorptiometry (DEXA; Discovery DXA Systems, Hologic Inc., Bedford, MA, US) scan to determine regional and whole-body composition (fat and fat-free mass) after fasting overnight. During analysis, participants lay in a supine position with their feet placed shoulder width apart. By the end of preliminary testing, participants were informed with instructions on how to safely ambulate on crutches prior to immobilization phase by a trained physiotherapist. The leg to be immobilized was randomly selected and counterbalanced for left/right. Then the immobilized leg was locked at a 30° angle of flexion to prevent participants from performing weight baring exercises and covered in anti-tamper tap to ensure it remained in place during 7-days period of immobilization. Participants were asked to remove the leg brace overnight and complete ankle exercise to minimise potential risks. After the 7-day immobilization phase, participants reported to laboratory following a 10 h overnight fast for repeat DEXA scanning immediately after removing the knee brace. Skeletal muscle biopsy samples were obtained from the quadriceps of immobilized (IMMOB) and non-immobilized control legs (CTRL). Post-immobilization quadriceps muscle isometric strength was measured the day after muscle biopsy sampling for participant comfort.

4.2.4 Human Skeletal Muscle Sample Collection

A portion of the muscle biopsy samples (~20mg) were used for immunofluorescence microscopy analysis of CreaT proteins were collected from 15 young healthy individuals

(characteristics presented in Table 4.1). Skeletal muscle biopsies were obtained from the *vastus lateralis* using the Bergstrom percutaneous needle biopsy technique, under local anaesthesia. Samples were cleaned using sterile saline to remove excess blood and dissected free of any visible fat, collagen and connective tissues. Biopsy tissue was then placed in Tissue Tek OCT compound and frozen in liquid nitrogen-cooled isopentane before being stored at -80℃ freezer until experimental analysis.

4.2.5 Human Skeletal Muscle Immunofluorescence Microscopy

Embedded muscle samples were fixed in front of a microtome blade (Bright 5040, Bright Company limited, Huntingdon, England) and cryosections (10μm) were collected onto room temperature uncoated glass slides (Thermo Scientific, Hungary).

4.2.5.1 CreaT Intensity

Sections were fixed at room temperature in 4% paraformaldehyde (PFA) solution (Fisher, UK) for 10 min and then washed 3×5 min in phosphate buffered saline (PBS) with 0.2% Tween (PBS-T). Sections were then blocked in 5% normal goat serum (Invitrogen, UK) in 1% bovine serum albumin solution (1% BSA) for 1 h at room temperature and then incubated with anti-SLC6A8 antibody (ab62196, abcam, 1:150, Cambridge, UK) and dystrophin (mouse IgGα, 1:100) mixed antibody in 1×PBS at 4℃ overnight. On following day, sections were washed for 3 × 5 min in 1× PBS-T and secondary antibodies were then applied to sections for 120 min at room temperature. After secondary antibody incubation, Sections were then left to dry for ~10 min and mounted with 20ul Mowiol ® 4-88 (SIGMA-Aldrich, Gillingham, UK) and covered with glass coverslips. Slides were left to dry overnight in a dark place before capturing images. Primary and secondary antibodies, experimental dilutions and other details are

provided in Chapter 2.

4.2.5.2 Muscle Fibre Morphology

Sections were incubated with Triton X 100 (at 0.02% concentration in 1×PBS) for 5 min and then blocked in 5% normal goat serum (Invitrogen, UK) in 1× PBS for 90 min at room temperature. Sections were incubated with MHCI (IgG2b, BAF8, DSHB, Iowa, US) and MHCII (IgG1, SC.71) mixed primary antibodies (in 1×PBS) at room temperature overnight. The following day, sections were washed for 3 × 5 min in 1×PBS-T and incubated in secondary antibodies and WGA mixed solutions in 1×PBS for 90min at room temperature. Following secondary antibody incubation, sections were then washed 2 × 5 min with PBS-T and 1 × 5 min with PBS and left to dry. Primary and secondary antibodies, experimental dilutions and other details are provided in Chapter 2.

4.2.6 Immunofluorescence Microscopy Image Capture

Wide-field image capture was completed using a Nikon E600 with a 40×0.75 numerical aperture objective. Images per area were captured under three colour filters achieved by a SPOTRT KE colour shot CCD camera (Diagnostic Instruments Inc., MI, USA), illuminated by a 170 W Xenon light source. For image capture, the Texas-Red (540-580mm) excitation filter was used to capture signals of dystrophin (sarcolemma), MHCI (Type I fibres). FITC (465–495 nm) excitation filter were used to capture signals of CreaT or MHCII (Type II fibres). DAPI UV (340–380 nm) filter was used to view WGA-350 (cell membranes) signals and stains in blue. Wide-field images for CreaT were obtained using 40x objective and muscle fibre types were obtained using 20x objective. All images processing and analysis were carried out in Image Pro Plus 5.1 (Media Cybernetics, MD., USA). CreaT fluorescence intensity was used to

identify the abundance of CreaT protein, as described in chapter 2.

4.2.7 Calculations

Ring area mean integrate density was used to identify CreaT protein content intensity around cell membranes. CreaT intensity and density are present by using a relative unit of measurement to show the ratio of amount of substance, refer as arbitrary unit (AU). Details of the calculations are provided in Chapter 2.

4.2.8 Statistics

All analyses were carried out using SPSS, version 22. Paired student T-test was used to compare variables between control and immobilised legs in same participant i) total intramuscular CreaT content, ii) total CreaT protein intensity, and iii) CreaT co-localisation. A repeated measurement was used to compare i) fibre CSA and fibre type composition, ii) CreaT protein mean intensity in fibre type specific manner and iii) ring and centre area intensity. A p value descriptive data was generated for all variables and the significance levels were set at $P<0.05$. Data are reported as Mean ± SD. Prism 8 (GraphPad Software, California, US) were used to draw the graphs.

4.3 Results

4.3.1 Muscle Morphology and Strength

Muscle morphology and strength data are presented in Table 4.1. Isometric strength in IMMOB was significantly reduced at post- compared with pre-immobilization (Pre: 698Nm ± 213 *vs*. Post: 502Nm ± 219; $P < 0.01$). No significant difference in isometric strength was found

between pre and post immobilization in CTRL (Pre: 667Nm ± 188 *vs.* Post: 586Nm ± 250, P = 0.067). Leg fat free mass was significantly lower following immobilization in IMMOB (Pre: 9.35 kg ± 1.27 *vs.* Post: 9.01kg ± 1.58, P < 0.01). No significant difference in leg lean mass was found in CTRL between pre and post intervention (Pre: 9.15kg ± 1.23 *vs.* 9.15kg ± 1.30, P = 1.00). Type II muscle fibre cross section area (CSA) was significantly smaller in IMMOB compared to CTRL (5561μm^2 ± 1865 vs. 6320 μm^2 ± 1597, respectively; P = 0.028). No significant difference in Type I muscle fibre CSA was found between IMMOB and CTRL (4700 μm^2 ± 1233 vs. 5425 μm^2 ± 1253; P = 0.07). The proportion of Type II fibres in the vastus lateralis was greater Type I fibres in IMMOB and CTRL limbs (P<0.01), with no difference in the proportion of Type I to II fibres between limbs.

Table 4.1 *Participants muscle morphology and isometric strength characteristics.*

	CTRL (N=15)		**IMMOB (N=15)**	
	Pre	Post	Pre	Post
Knee extensor isometric strength (kg)	667 ± 188	586 ± 250	698 ± 213	502 ± 219**
Leg fat free mass (kg)	9.15 ± 1.23	9.15 ± 1.30	9.35 ± 1.27	9.01 ± 1.58**
Type I fibre CSA (μm^2)	---	5425 ± 1253	---	4805 ± 1256
Type II fibre CSA (μm^2)	---	6320 ± 1597	---	5561 ± 1804*
Type I fibre (%)	---	40 ± 8	---	43 ± 9
Type II fibre (%)	---	60 ± 9	---	57 ± 9

Data are presented as Mean ± SD. Symbols indicate significantly different from CTRL, significant difference was set at $P < 0.05$, * indicates $P < 0.05$, ** indicates significant difference $P < 0.01$.

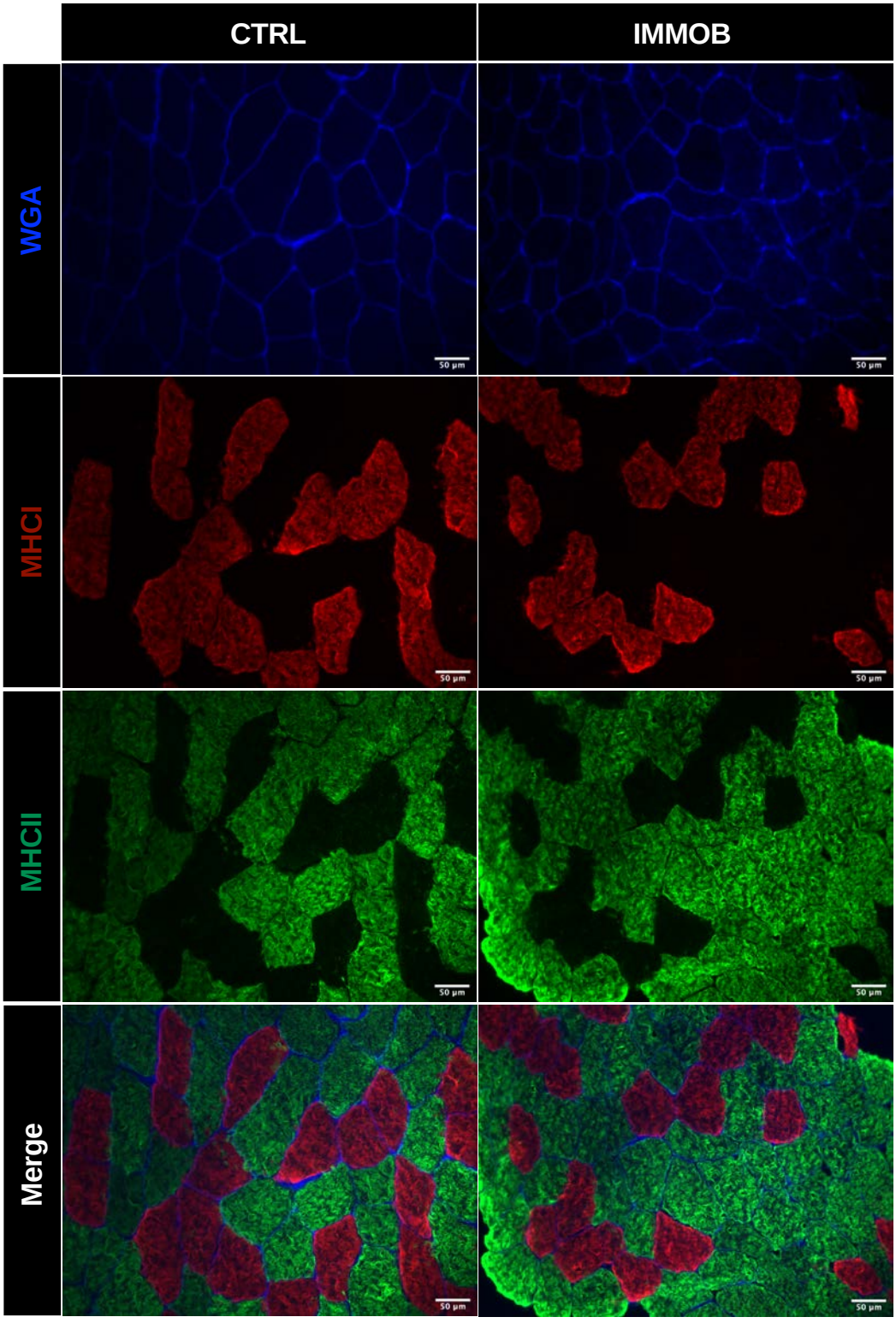


Figure 4.1 *Immunofluorescence microscopy images of human skeletal muscle fibre types. Sections marked with MHCI (Type I fibres) (Red stain), MHCII (Type II fibres) (Green stain)*

and WGA (Blue stain). WGA was used to mark cell membranes. Magnification of images are 20× objective, Scale bars are 50 µm.

4.3.2 Skeletal Muscle CreaT Protein content

Total CreaT content was normalised to fibre CSA to obtain per area CreaT protein content intensity (Figure 4.2 and 4.3). Results showed that CreaT protein intensity was significantly greater in IMMOB than in CTRL (CTRL 34.84 ± 6.45 *vs.* IMMOB 41.94 ± 7.51, P = 0.018).

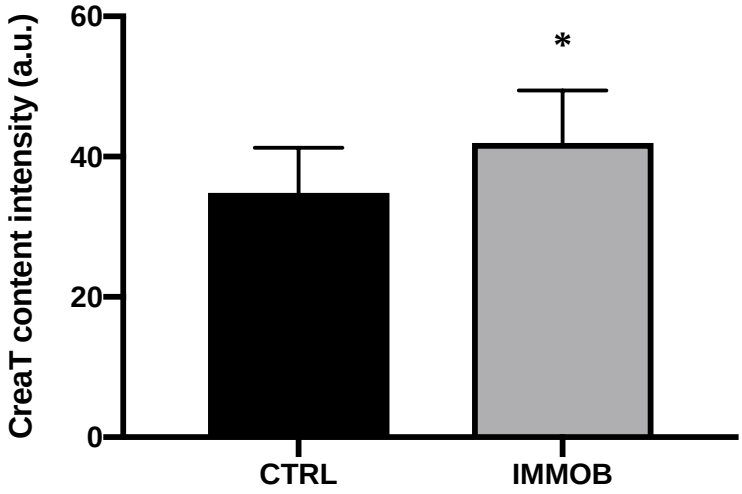


Figure 4.2 *Human skeletal muscle CreaT protein mean intensity in CTRL and IMMOB. Repeated measurements were used to analysis the difference between CTRL and IMMOB legs. Values are mean ± SD. Significance was set at P < 0.05. * shows greater CreaT content P < 0.05. a.u. means arbitrary unit.*

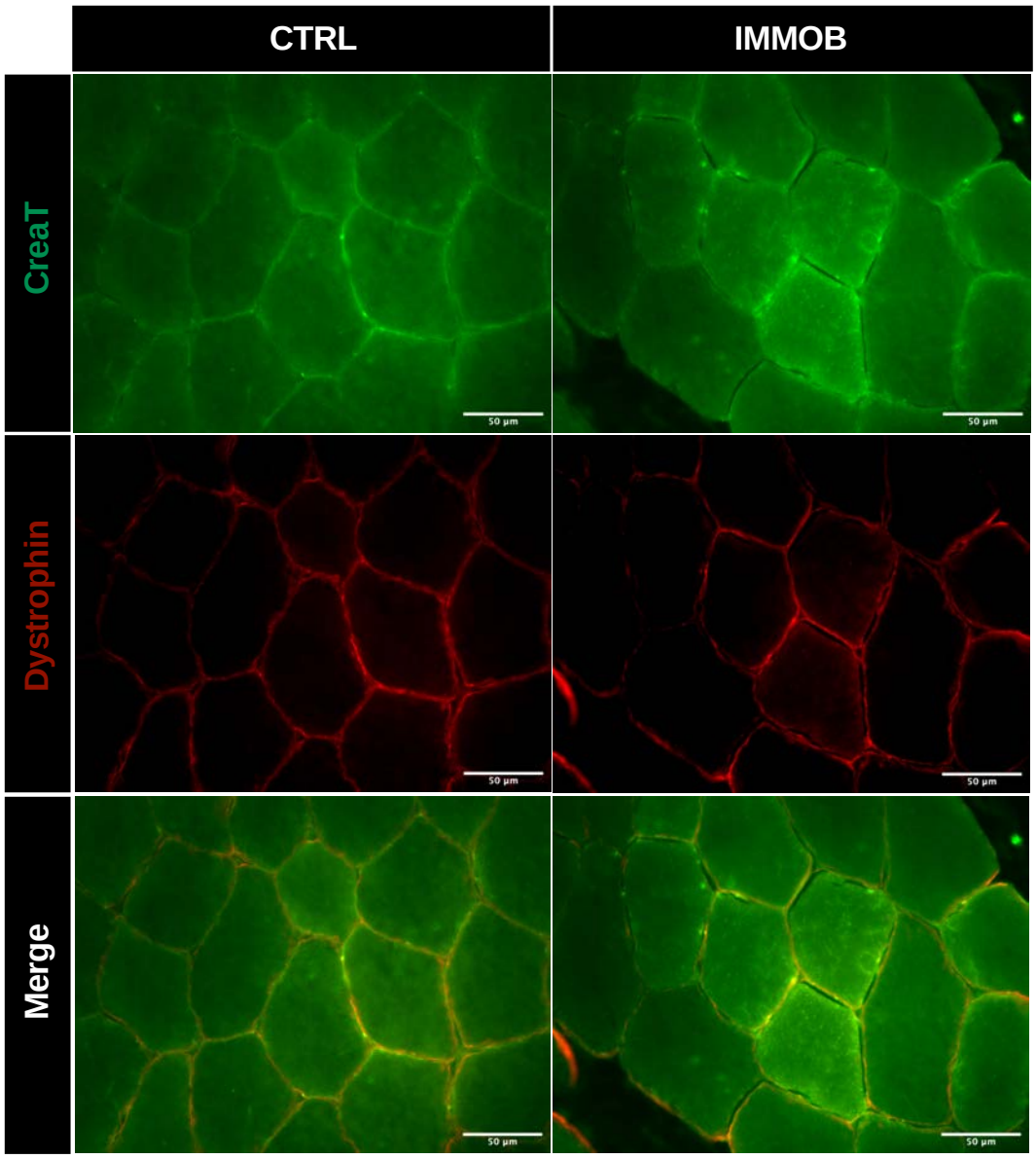


Figure 4.3 *Immunofluorescence microscopy images of CreaT protein intensity in CTRL and IMMOB. Sections were marked with CreaT protein (Green stain), Dystrophin (Red stain). Dystrophin was used to mark cell membranes. Magnification of images are 40× objects. Scale bars are 50µm.*

4.3.3 Fibre Type Specific CreaT Protein Content

CreaT protein content normalised to fibre area, total intramuscular CreaT protein IntDensity was divided by fibre CSA in CTRL and IMMOB legs to obtain per area CreaT density (**Figure 4.4**). Type I muscle showed significantly greater CreaT protein intensity values compared with Type II muscle fibres in both CTRL and IMMOB (CTRL: Type I 47.54 ± 8.00 *vs.* Type II 41.32 ± 5.27, $P < 0.01$; IMMOB: Type I 56.61 ± 12.34 *vs.* Type II 48.90 ± 9.73, $P < 0.01$). IMMOB Type I muscle fibres CreaT protein intensity (56.61 ± 12.34) was significantly greater than IMMOB Type II and CTRL Type II fibres and tended to be greater compare with CTRL Type I fibres. (IMMOB Type I vs. IMMOB Type II, $P < 0.01$; IMMOB Type I vs. CTRL Type II, $P = 0.002$; IMMOB Type I vs. CTRL Type I, $P = 0.074$)

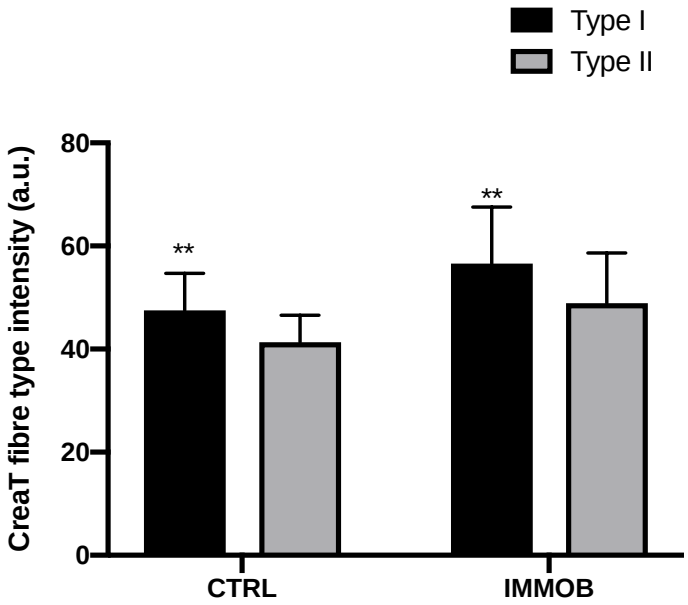
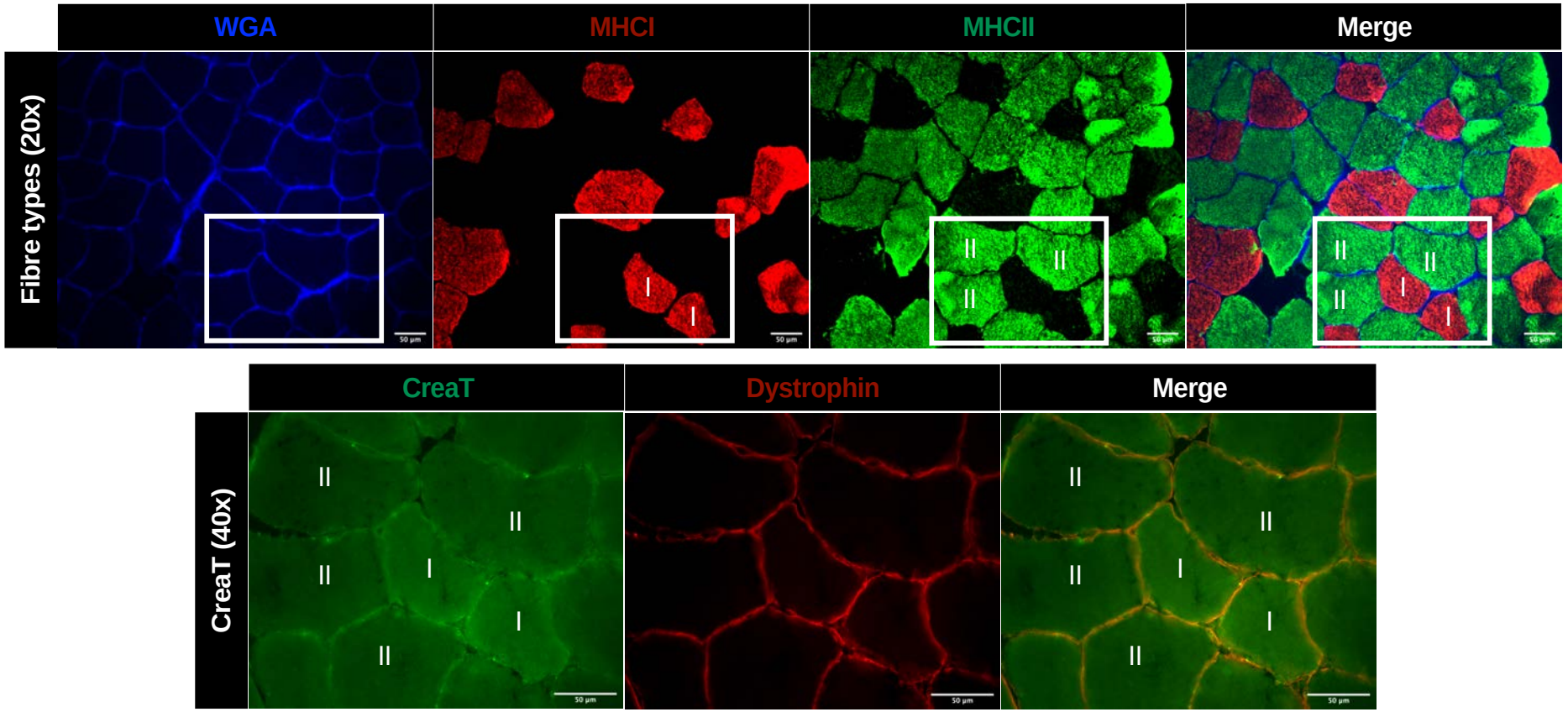


Figure 4.4 *CreaT protein intensity in fibre type specific manners in CTRL and IMMOB. Values are set at Mean ± SD. Repeated measurements was used to indicate significant difference of CreaT protein intensity between Type I and Type II muscle fibres, and between CTRL and IMMOB. Significance was set as $P < 0.05$. * shows significantly greater CreaT intensity Type I vs. Type II fibre within the same limb, ** shows significance $P < 0.01$ within the same limb;*

A

CTRL


WGA
MHCI
MHCII
Merge
Fibre types (20x)
I
II
CreaT
Dystrophin
Merge
CreaT (40x)

B

IMMOB

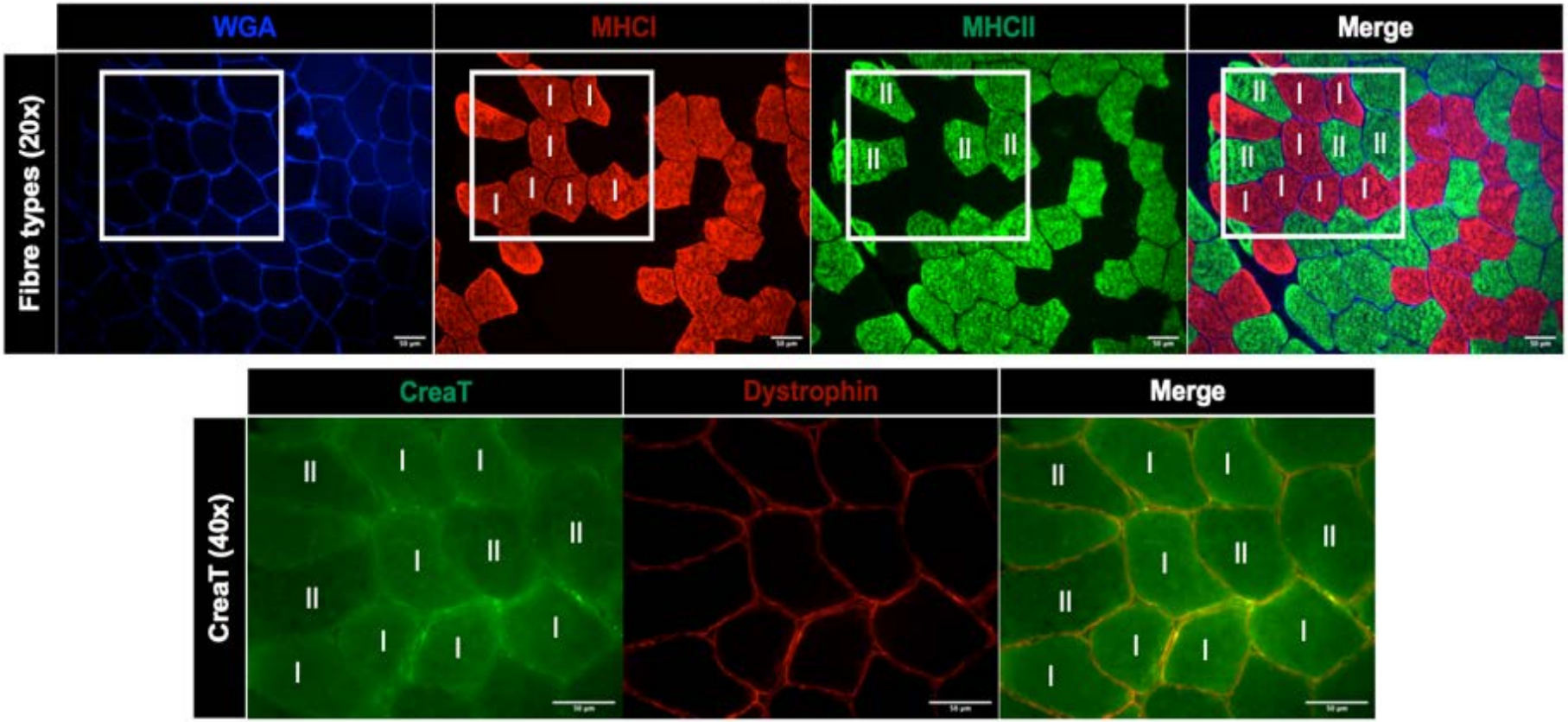

WGA
MHCI
MHCII
Merge
Fibre types (20x)
CreaT
Dystrophin
Merge
CreaT (40x)

Figure 4.5 *Immunofluorescence microscopy images of fibre type specific CreaT intensity in CTRL and IMMOB. Serial sections were stained for MHCI (Red stain), MHCII (Green stain), and WGA (Blue stain) on top panels of group* ***A (CTRL)*** *and* ***B (IMMOB)*** *with 20x magnification, stained for CreaT protein (Green stain) and dystrophin (Red stain) on bottom panels of group* ***A*** *and* ***B*** *with 40x magnification. Scale bars of images are all set at 50μm. These serial sections stain represent the fibres in muscle fibre type stain sections (areas in white box) equivalent the fibres in section stained for CreaT protein intensity. The quantification was carried out using dystrophin stain to mark cell membranes.*

4.3.4 CreaT Content in Specific Areas (ring and centre)

Ring and centre area CreaT intensity were applied to determine the CreaT sub-cellular distribution (**Figure 4.5**). In both CTRL and IMMOB, ring area CreaT protein mean intensity was significantly greater compared with the centre area (CTRL: Ring area 50.41 ± 7.89, Centre area 43.67 ± 6.81, $P < 0.01$; IMMOB: Ring area 59.29 ± 11.85, Centre area 51.93 ± 10.81, $P < 0.01$). When comparing CreaT mean intensity between CTRL and IMMOB, ring area CreaT mean intensity was significantly greater in IMMOB than CTRL (CTRL: 50.41 ± 7.89 *vs.* IMMOB: 59.29 ± 11.85, $P < 0.01$). Centre area CreaT protein mean intensity was significantly greater in IMMOB group compare to CTRL (CTRL: 43.67 ± 6.81 *vs.* IMMOB: 51.93 ± 10.81, $P < 0.01$) (**Figure 4.6**).

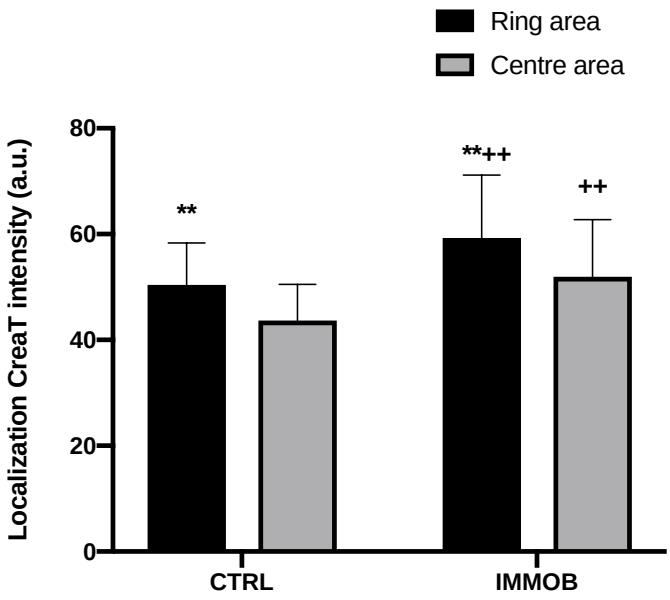


Figure 4.6 *CreaT protein intensity in ring and centre areas. Values are set at Mean ± SD. Repeated measurements was used to indicate significant difference of CreaT protein intensity in different areas (Ring and Centre area), and between CTRL and IMMOB conditions. Significance was set as P < 0.05. * shows significantly greater CreaT intensity in ring vs. centre area within the same limb, ** shows significance P < 0.01 within the same limb; + shows significance of P < 0.05 in same areas between limbs, ++ shows significant difference of P < 0.01 in same areas between limbs.*

4.3.5 Fibre Type CreaT Content in Specific Area

CreaT intensity in ring and centre areas were normalised into per CSA area content in different fibre types (Figure 4.7). In the ring area, within same limbs, CreaT protein intensity was significantly greater in Type I compared with Type II muscle fibres in both CTRL and IMMOB (CTRL: Type I 53.48 ± 7.99 vs. Type II 47.34 ± 6.75, P = 0.004, IMMOB: Type I 62.38 ± 12.34 vs. Type II 56.19 ± 10.92, P = 0.002). No significant difference in ring CreaT intensity was found between CTRL and IMMOB (**Figure 4.7A**). In the centre area, CreaT protein intensity was greater in Type I compared to Type II muscle fibres in both CTRL and IMMOB

(CTRL: Type I 46.65 ± 7.07 vs. Type II 40.69 ± 5.26, P = 0.02, IMMOB: Type I 55.85 ± 10.80 vs. Type II 48.01 ± 9.67, P <0.01). No significant difference in centre CreaT content was observed between CTRL and IMMOB (**Figure 4.7B).**

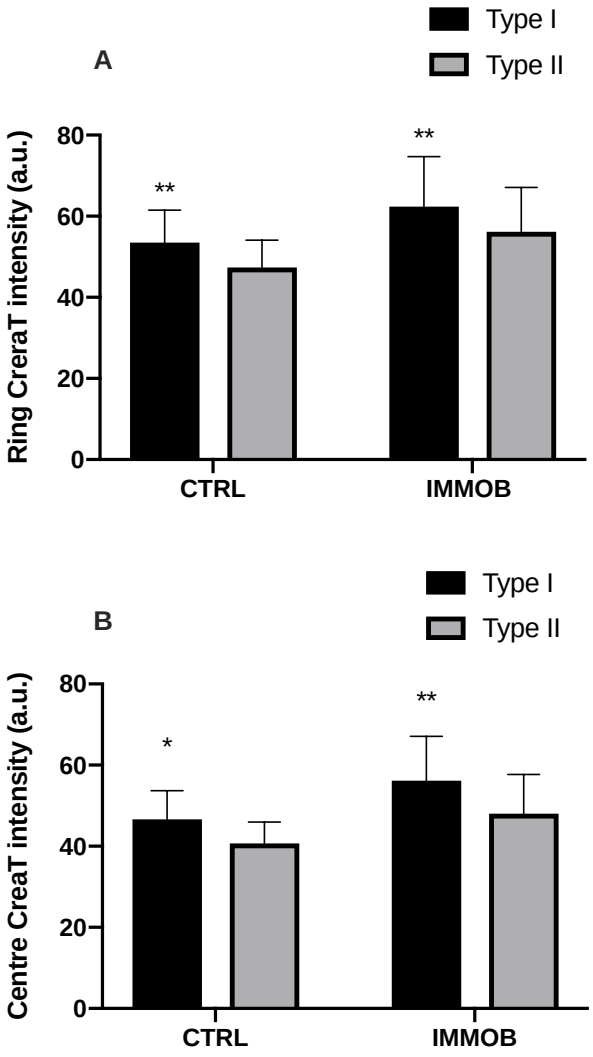


Figure 4.7 *CreaT protein intensity in specific regions in Type I and Type II muscle fibres. A represents CreaT protein content in per ring area in different muscle fibre types,* ***B*** *represents CreaT protein content in per centre area in different fibre types. Repeated measurements were used to indicate significant difference of CreaT protein intensity in different areas (Ring and Centre area), in different muscle fibre types and between CTRL and IMMOB conditions. Significance was set as P < 0.05. ** shows significance CreaT protein content P < 0.01 within the same limb.*

4.4 Discussion

The present study is the first to investigate alteration in intramuscular CreaT protein during a short-term disuse in humans. Our findings showed that 7-days of unilateral leg immobilization (via knee bracing) induced a reduction in skeletal muscle fat free mass and isometric strength in human skeletal muscles, which corroborates with the findings in previous immobilization studies (1, 17, 20). In addition, muscle fibre CSA was ~11% lower in Type I muscle fibres and ~12% lower in Type II muscle fibres from immobilized vs. non-immobilized limbs (IMMOB and CTRL, respectively). Again, this observation is consistent with that of Suetta *et al.* who reported an ~10% reduction in muscle fibre CSA in young individuals after 4 days of limb immobilization (30) as well as other short-term bed rest studies (15).

The immunofluorescence microscopy method optimised in Chapter 2 and further developed in Chapter 3 of this thesis has been used herein to investigate the effect of immobilization on fibre-type CreaT protein expression and sub-cellular distribution for the first time in humans. Given evidence that periods of disuse may adversely affect cellular energetics in humans, we hypothesized that CreaT protein expression would be lower in an IMMOB vs. CTRL. In contrast to our hypothesis, intramuscular CreaT protein content was actually greater in immobilized vs. non-immobilized muscle, indicative of a potential increase in CreaT with disuse. Although the mechanisms through which immobilization initiated an apparent increase in intramuscular CreaT are unclear , this result may be explained by potential reductions in PCr / Cr levels in muscle, as reported elsewhere with disuse-induced muscle atrophy (11, 22, 29). Considering the reduction in locomotor activity, mechanical tension and energy expenditure that typically occurs during immobilization, energy-related signalling pathway may initiate an up-regulation of CreaT expression as a compensatory mechanism to maintain sufficient intramuscular Cr uptake for cellular energetics, although this contention requires further investigation.

In human skeletal muscle, Cr pool size is relatively constant and the process of Cr uptake via CreaT is suggested to be a saturable process. As such, the amount of intracellular Cr storage may be a 'modulator' to regulate the content and distribution of CreaT protein. This notion is in agreement with evidence that CreaT protein is extremely sensitive to the amount of intramuscular Cr and is up-regulated when Cr is reduced (7, 28). In addition, a decreased Cr uptake rate has been associated with a lower creatine and sodium concentration in rat muscle (27). This notion may also be indirectly supported by evidence from Cr supplementation studies, which suggest that short-term creatine loading (~20g/day or 0.2-0.3/kg/day over 5-7 days) can significantly improve intracellular Cr concentration in different populations (7, 13, 14, 18), after which lower Cr maintenance doses over the following few weeks will not further increase intracellular Cr levels (5, 14). The less effectiveness of Cr supplementation during maintenance dose may due to the Cr storage level in skeletal muscle appears to reach saturable level. Therefore, Cr supplementation is potentially be more effective in the populations that suffering low intramuscular Cr levels. Considering the lifestyle associated changes in older individuals, intake a maintenance dose of Cr supplementation may be benefit to ageing muscle and functions. Additionally, prolonged Cr supplementation (i.e. >several weeks) does not continually increase the intramuscular TCr pool (14). Thus, circulating creatine concentrations following prolonged supplementation may provide a signal to skeletal muscle that intramuscular Cr storage is saturated. Therefore, only certain amount of CreaT protein may be required to support the task of transporting Cr to the intracellular space. Muscle may gradually adapt to an environment of sufficient Cr over time (37) and potentially lead to a down-regulation of CreaT. This potential mechanisms of Cr modulation has also been supported by work in mice, which demonstrates that prolonged Cr supplementation decreased the degree of muscle mass and strength improvement and down-regulated CreaT protein expression (4, 12). At present, the specific promoter of CreaT gene expression and down-regulation of

intramuscular Cr level remains unclear. However, the AMPK molecular pathway has been well accepted to play roles in regulating CreaT activities due to its responsiveness to ratios of Cr: PCr (9, 38) and warrants further investigation. In summary, skeletal muscle Cr uptake occurs specifically via CreaT, and the expression of CreaT may be modulated by intramuscular Cr and/or PCr levels. Immobilization-induced muscle atrophy may decrease the intramuscular Cr pool and disrupt energy homeostasis, subsequently modulate CreaT expression (potentially through the AMPK signalling pathway). The mechanistic regulation of the observed greater CreaT in IMMOB vs. CTRL requires further complementary analysis of intramuscular high-energy phosphagens (Cr/PCr/ATP) and AMPK signaling, that were unavailable for this study due to limited tissue availability.

In terms of fibre-type specific CreaT protein expression, we report that Type I muscle expressed greater CreaT protein content than Type II muscle fibres. This finding is consistent with earlier work (3, 24, 31) and supports an inverse relationship between Cr content and CreaT expression, in which Type I fibres may have a greater capacity for Cr uptake compared with Type II fibres. In order to explore the difference in CreaT between IMMOB and CTRL, we analyzed CreaT content at the fibre-specific level. The idea that the greater CreaT content in IMMOB vs. CTRL might be explained by preferential Type II fibre atrophy and an increase in the relative proportion of Type I fibres (i.e. a shift to a 'slow' muscle phenotype) is not entirely supported by our findings. Specifically, whilst we report a significantly lower Type II fibre CSA in IMMOB vs. CTRL, there was a trend for a lower Type I fibre CSA and no difference in the proportion of Type I and II fibres between limbs. Given the relationship between muscle morphology and TCr (6, 7), a decrease in muscle fibre CSA may be a key factor of the reduction of intramuscular Cr content with disuse. Although there are differences in PCr content between Type I and Type I fibres, the significantly lower Type II fibre CSA and trend for a lower Type I fibre CSA in IMMOB vs. CTRL, indicative of a reduction in fibre size with disuse, may have

reduced intramuscular PCr content, as has been reported elsewhere previously (11). Subsequently, this disuse-induced impairment in high-energy phosphagens and energy homeostasis may have promoted an upregulation of CreaT expression. Indeed, whilst we did not detect fibre-specific differences in CreaT content between IMMOB and CTRL limbs, significant differences between limbs became apparent when all CreaT content of all fibres was combined.

Although we were unable to detect a fibre-specific differences in CreaT content to explain the greater CreaT content in IMMOB vs. CTRL, we applied a novel immunofluorescence microscopy imaging technique to determine whether there were differences in the sub-cellular localisation of CreaT between limbs. Consistent with previous studies (23, 24), our findings showed that CreaT was distributed predominantly at the cell membrane region in both Type I and II fibres. For the first time, we also showed that CreaT content was greater at the membrane and throughout the rest of the cell in IMMOB vs. CTRL. There are a number of possible reasons to explain why CreaT would associate with cell membranes, not least of which is the requirement to support intramuscular Cr uptake after energy depletion (e.g. following high intensity exercise contractions (32)). Furthermore, the predominant membrane localisation of CreaT is also consistent with its purported role as a neurotransmitter (10, 25, 26). When considering fibre-type specific differences in CreaT expression together with CreaT sub-cellular localisation, the greater expression of CreaT in Type I fibres may due to the different functions of Type I (oxidative) and Type II (glycolytic) fibres. Thus, future investigations should aim to understand the role of CreaT in substrate utilization in distinct fibres (i.e. localisation with mitochondria and lipid droplets) and changes in the sub-cellular localisation of CreaT in response to altered cellular energy and phosphagen status (i.e. following demanding exercise and/or supplemental creatine loading).

4.5 Conclusion

In summary, this study aimed to investigate the effect of short-term disuse on muscle fibre-specific CreaT protein expression and sub-cellular distribution in human skeletal muscle. We report that 7-days of immobilization resulted in a substantial loss of skeletal muscle mass, fibre area and isometric strength. In addition, immobilization apparently increased CreaT protein content. Although we cannot be certain, we speculate that the potential unbalanced energy homeostasis induced by muscle atrophy may have initiated the expression or up-regulation of CreaT to attenuate cellular energy depletion. Our findings also show that CreaT is distributed mainly at the cell membrane with a greater expression in Type I over Type II muscle fibres, potentially related to its role in Cr uptake. Finally, the increase in intramuscular CreaT content with immobilization was evident at the cell membrane and throughout the whole cell. Thus, this study is the first comprehensive investigation of the impact of disuse-induced muscle atrophy on CreaT protein expression. These findings provide an evidence for future research to examine whether fibre-specific CreaT expression/activity is a rate-limiting step in the muscle anabolic response to exercise and supplemental creatine interventions that may attenuate disuse induced muscle atrophy. As CreaT is the essential modulator by which Cr enters muscle cells, maintenance of CreaT protein content and capacity, in addition to cellular energy homoeostasis could be an important point of intervention.

Disclosures

The authors have no conflicts of interest to declare.

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Authorship statement

All authors gave their final approval of the version of the article to be published. DL and LB designed the study. LB and SE organized and carried out the clinical experiments with the assistance of DL. DL, SE and LB performed all data analyses. DL, SE and LB performed the statistical analysis of the data. DL and LB wrote the manuscript together. DL and LB are the guarantors of this work and take responsibility for the integrity and accuracy of the data analysis.

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Chapter 5

General Discussion

5.1 Implications of Thesis

The importance of skeletal muscle plasticity in health and disease is becoming ever more clear through growing research efforts. The weight of evidence supports the key role of cellular Cr metabolism in the maintenance of skeletal muscle health. To date, the role of CreaT in modulating Cr metabolism in skeletal muscle are yet to be fully understood, and the last investigation on the role of skeletal muscle CreaT sub-cellular function was conducted more than 15 years ago. In this thesis, we developed new techniques and generated proof of concept data to show how CreaT is modulated in models of muscle ageing and disuse (i.e. accelerated ageing). In this regard, the work completed over the course of this thesis provides a framework for future studies on skeletal muscle morphology and Cr metabolism during disuse. Alterations in CreaT may explain muscle deterioration in conditions of ageing and disuse atrophy, and the therapeutic interventions to support muscle health may well need to be targeted to CreaT.

5.2 Novel Findings in Thesis

5.2.1 Detection of CreaT via Immunofluorescence Microscopy Techniques

CreaT protein activity is important for modulating muscle Cr metabolism in response to ageing and physical activity. The measurement of gene expression, mRNA and protein content have been the most widely used techniques to investigate CreaT (43, 49, 50). However, CreaT only function at the cell membrane, therefore, muscle homogenate measurements may lack the precision to explain the activities of CreaT in response to intracellular Cr alterations in different situations. The immunofluorescence microscopy method could be therefore providing a stronger evidence to determine CreaT content and activities in response to Cr uptake. Immunofluorescence microscopy data on CreaT have been reported in both rodent models and

human muscle tissue (29, 30). This thesis applied a series control tests to validate and optimize a new method of immunofluorescence and immunoblotting analysis, before investigating CreaT content and sub-cellular localisation in human muscle tissues (Chapters 3 and 4). Both peptide blocking and CreaT KO tissue controls confirmed that the CreaT antibody we used in this thesis was specifically targeted to CreaT protein. Our immunofluorescence method indicated that the immunofluorescence methods used by others previously lacked appropriate primary antibody controls when applying mixed antibody 'cocktails' in their analysis. MHCI primary antibody led to unspecific binding of the CreaT protein signal, which erroneously increased the intensity of the CreaT protein stain in Type I muscle fibres. Therefore, extending on the previous CreaT staining methods, in **Chapter 2** of the present thesis, we stained CreaT protein and fibre types separately, and used a series of muscle sections to determine fibre-type specific CreaT content. Our optimized immunofluorescence microscopy technique provided a more accurate and reliable way to determine CreaT protein distribution and sub-cellular localisation in human skeletal muscle. Following these technical developments, in **Chapter 3** of this thesis, we found that CreaT protein was significantly greater Type I compared to Type II fibres in older individuals, with no detectable difference in younger individuals. Furthermore, CreaT was expressed predominantly at the cell periphery in both young and older individuals (***Figure 5.1*A**). In **Chapter 4** of this thesis, CreaT protein content was significantly greater in muscle following 7-days of immobilization (IMMOB) compared with the non-immobilized contralateral control limb (CTRL), in line with reductions in fibre size and strength in IMMOB. CreaT was greater in Type I compared to Type II muscle fibres in both CTRL and IMMOB group, and was expressed predominantly at the cell periphery in both limbs. The greater CreaT content in IMMOB vs. CTRL may be explained by a trend for an increase in Type I fibres (***Figure 5.1*B)**. There are a number of possible explanations for the discrepant fibre-type CreaT

content in young individuals in Chapter 3, where we saw no difference in fibre-type CreaT, and Chapter 4, where we saw greater Type I fibre CreaT in younger individuals. **Chapter 3** included a total of 12 samples (six young and six older) from male and female volunteers, which may explain the variability in CreaT content in the young, which is presented in ***Figure 5.1*****A**. In contrast, **Chapter 4** included a total of 26 samples from males only (thirteen CTRL and thirteen IMMOB) for analysis. Therefore, despite this discrepancy, we believe our findings are in general agreement with earlier work (5, 29, 30), demonstrating that CreaT protein content differs between fibre-types in human skeletal muscle. Future research based on CreaT protein investigation should apply a caution when estimating sample size requirements.

The specific sub-cellular region calculation method developed herein offers a new insight to study CreaT protein localisation in muscle fibres. By using this novel method, skeletal muscle CreaT protein was found to localize predominantly at the cell periphery, as identified by greater ring area intensity compared to centre area intensity in all groups (**Chapter 3** and **Chapter 4**).

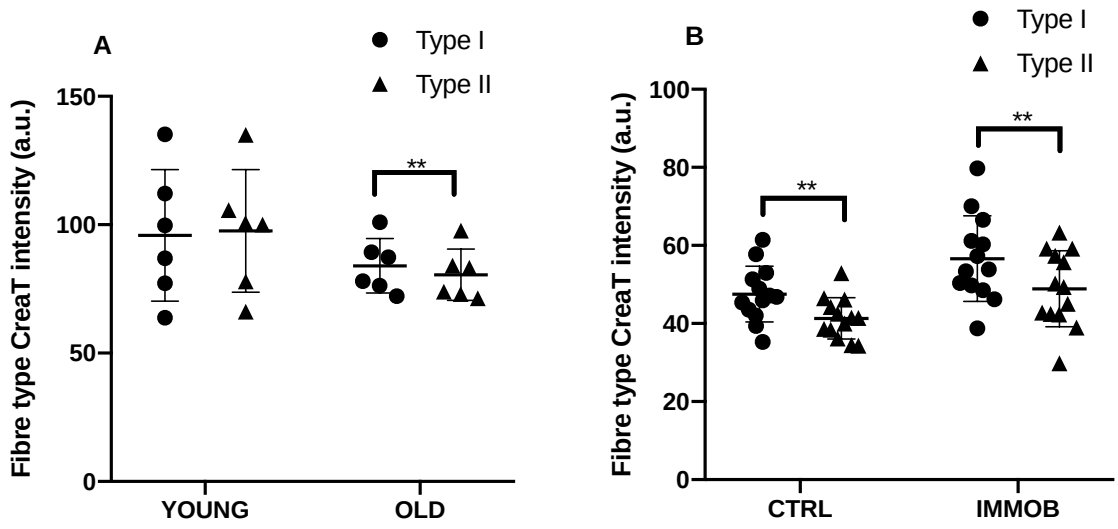

Figure 5.1 Fibre type specific CreaT Intensity from all volunteers involved in this thesis.

5.2.2 The Effect of Ageing in Muscle CreaT Expression and Cr Metabolism

Ageing is associated with various impairments in skeletal muscle cellular regulation that results in a high risk of disability, impaired physical function, hospitalisation, and even mortality (3, 27, 42). These cellular impairments are often directly correlated with sarcopenia and frailty in old population (11). Skeletal muscle frailty is a complex geriatric syndrome that habitually increases skeletal muscle weakness to stressors, leading to a declined physiologic storage that cause impairment of cellular homeostasis (11, 31). Cellular metabolic homeostasis is generally associated with steady energy turnover (22). PCr/Cr has been shown to play important role in sustaining energy homeostasis (20, 48) and cellular Cr uptake is highly depended on its specific CreaT. Based on previous studies, ageing individuals have been found to have lower intramuscular PCr and/or Cr concentration compared to healthy young individuals, especially in the quadriceps (9, 38, 41), an observation that was confirmed in our preliminary findings in **Chapter 3**. Further to this, our data showed a significantly greater intramuscular PCr concentration in young compared with older muscle, which was accompanied with a significantly higher creatine kinase (CK) content in young compared with old. These findings are in line with previous research showing that PCr content is lower in older vs. young healthy skeletal muscle (4, 28). Meanwhile, we were unable to detect any significant difference of either Type I or Type II muscle fibre size between young and old individuals. As such, the suggestion that the reduced PCr content in ageing muscle may due to Type II fibre atrophy (8), does not appear to explain our findings, although it should be noted that samples for analysis of PCr were obtained from a different cohort to those analyzed for fibre morphology and CreaT. Therefore, the lower PCr concentration present in **Chapter 3** and elsewhere, may not necessarily be driven by a reduction in muscle fibre size. It should also be noted that the participants in **Chapter 3** were in relatively good health, which may explain the absence of any clear morphological or fibre-type CreaT difference between young and older individuals.

Therefore, the potential decrease of PCr in ageing muscle could potentially be due to altered food intake (particularly meat), with lower food intake in older population has been previously reported (7, 26). Taken together, these findings suggest that intramuscular Cr metabolism may be altered by chronological ageing, whereas CreaT protein content remains relatively unaffected. This dissociation between Cr and CreaT protein may be explained by the unchanged energy homeostasis in these healthy old muscles. From the data, no changes were found in ATP levels between young and older muscles. Therefore, we may conclude that chronical ageing per se may not necessarily lead to a diminution of cellular energetics and homeostasis. Under conditions of cellular energy homeostasis, CreaT expression and/or translocation does not appear to be altered. This conclusion is partly in agreement with previous studies, in which Cr supplementation significantly increased intramuscular Cr concentration, whereas CreaT mRNA and protein content (in muscle homogenates) remained relatively unaffected (43). Beyond a scenario of healthy ageing, lower intramuscular PCr and/or Cr concentrations have closely linked to musculoskeletal disorders, cancer and neurological disorders.

Overall, balanced creatine metabolism is important for patients suffering from different diseases to attenuate the loss of muscle mass and physical function. To understand the mechanisms of CreaT regulation and its role in Cr metabolism may therefore help to improve intramuscular Cr levels through therapeutic interventions.

5.2.3 Disuse-induced Muscle Atrophy and CreaT

Skeletal muscle inactivity, mechanical unloading or disuse/immobilization occur as a consequence of illness or injury. This muscle disuse leads to a reduction in muscle mass, strength, functional capacity and a decline in cellular metabolic rate. During disuse, the loss in muscle fibre CSA is generally fast, with the mean rate of ~1.0% per day (34, 45), which is most

apparent at the onset of immobilization. In **Chapter 4**, we observed a decrease muscle fibre CSA and strength that was accompanied with a greater CreaT protein content after 7-days of immobilization. No direct evidence has been shown to explain the potential mechanistic link between muscle atrophy and Cr metabolism. However, the findings in Chapter 4 could be explained by the fact that disuse induces low energy requirements and alterations in appetite/hunger (47). Low energy usage during immobilization may alter cellular energy status and up-regulate CreaT in to generate more skeletal muscle Cr to restore energy homeostasis. In addition, muscle atrophy has been reported to associate with reduced intramuscular PCr / Cr levels (16, 28, 41). Taken together, these findings indicate that there may be a potential link between the alterations in CreaT content, reductions in intramuscular PCr concentration (reported elsewhere) and the loss of skeletal muscle mass during disuse (shows in ***Figure 5.2***). Prior to disuse, intramuscular PCr and CreaT are stable, therefore could provide sufficient energy to maintain muscle mass. Short periods of disuse (0-1 week) may lower cellular energy status, which may initiate signalling pathways that lead to a rapid muscle atrophy through impaired muscle protein synthesis rates and/or elevated breakdown. The impaired energy status during disuse may also initiate CreaT expression in order to increase Cr entry into muscle cells. During recovery from disuse, muscle reloading will likely restore cellular energy homeostasis. Therefore, sufficient CreaT protein may be required to assist in the restoration of intramuscular Cr levels. This mechanism is supported by the studies in which Cr supplementation was reported to disuse muscle atrophy in rats and humans (32). Furthermore, in **Chapter 4**, we observed a great sub-cellular location of CreaT protein at the cell periphery, which reflects the functional role of CreaT at cell membranes in transporting Cr from blood into skeletal muscle.

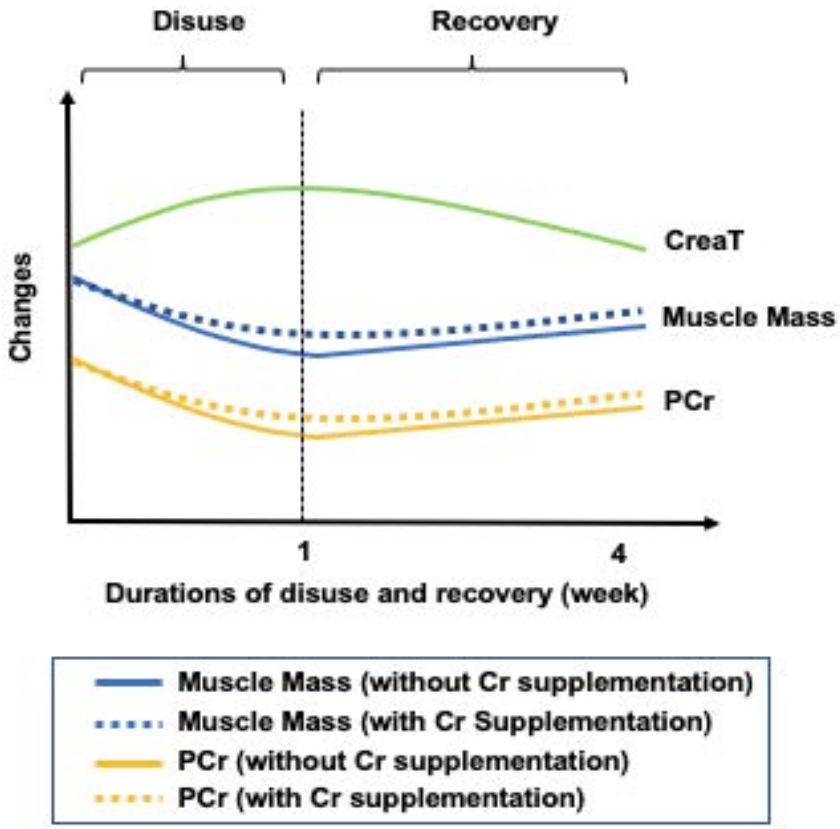

Figure 5.2 Theoretical schematic of the potential associated alterations of skeletal muscle atrophy, PCr and CreaT content during disuse, and the potential role of Cr supplementation.

5.3 The Role of CreaT Protein in Skeletal Muscle: An Updated Overview

The findings of this thesis generate important new knowledge of the regulation of CreaT expression and localisation in human skeletal muscle. To sum up the findings of CreaT distribution in this thesis, we observed that CreaT protein is not altered by ageing but is greater with muscle disuse-induced muscle atrophy. The initiation of muscle atrophy occurs in various scenarios of altered proteostasis and cellular energy status, including i) ageing; ii) disuse, such as immobilization and bet rest; and iii) chronic diseases, such as diabetes and acquired muscular disorders (13). Considering the implications of our findings in the context of these scenarios, we show that CreaT expression in human skeletal muscle can be identified in three different

conditions. Firstly, in healthy skeletal muscle, CreaT expression and sub-cellular localisation is consistent in young and older individuals, although larger sample size experiments are required to confirm this. This potential mechanism is in agreement with previous findings from Tarnopolsky, which reported that acute and moderate-term Cr supplementation did not alter CreaT mRNA or protein content in either young or older individuals (43). The second scenario is disused induced atrophy, such as physical inactivity, immobilization or bed rest, leading to a decline in postabsorptive and postprandial MPS (34) and the cellular metabolic environment. Up-regulation of CreaT expression in disuse may be initiated as a consequence of the changed cellular metabolic environment, as a means to recruit greater Cr entry in skeletal muscle to restore energy balance and normal metabolic processes. Finally, in disease related disordered muscles, CreaT function and expression may down-regulate by the impaired physiological environment (44). Although, no data were presented in this thesis. Based on previous findings, the reduction of CreaT content appeared after prolonged Cr supplementation ingestion in rat model (19), which suggested the effectiveness of physiological stimuli on the role of the modulation of CreaT expression. In addition, lower CreaT protein content accompanied with the declined Cr and/or PCr concentrations was generally appeared in various muscular disorder diseases (i.e. mitochondrial cytopathy, inflammatory myopathy) (21, 35, 44). These evidences may reflect the potential correlation between CreaT and muscular disease are as a consequence of altered physiological environment.

5.4 Limitations of Research

There are some limitations in this thesis. Firstly, the study in **Chapter 3** just included six young and six older individuals, with a small sample size, caution must be applied, as the findings may not represent the whole population, future research based on CreaT protein research should

involve more subject sample to analyse. Additionally, due to the analysis technique basis, we could not quantify the co-localisation of CreaT and eNOS and we did not observe a strong immune-reactivity between CreaT and eNOS. An analysis technique should be developed to analysis the co-localisation between CreaT and eNOS. Furthermore, both Chapter 3 and 4 are just provided baseline values, as no intervention (exercise or supplementation) were included in these two studies, in chapter 4, we just took a biopsy after immobilization (for comparison against the non-immobilized control leg). As no biopsies were taken before immobilization, we do not have the muscle fibre CSA values before immobilization and cannot comment on how this may have shifted. However, it is to be expected that immobilization would lead to a reduction in type II and, to a lesser extent, type I fibres, thereby a 'slowing' of skeletal muscle phenotype.

5.5 Future Directions

This thesis provides a novel insight of CreaT alterations in ageing and muscle disuse. We suggest that skeletal muscle CreaT expression is not altered in healthy ageing, but may be altered in ageing when significant atrophy is present (i.e. in inactive older adults). The reduction of PCr and CK levels in ageing may affect CreaT movement or capacity in response to alterations in cellular energy status (i.e. with Cr supplementation or depleting exercise), but may not alter CreaT content per se, which warrants further investigation. Furthermore, we do not know the specific modulating mechanisms underlying the regulation of CreaT expression. In order to better understand the role of CreaT in cellular Cr metabolism and its functions in skeletal muscle physiology, more investigations are required. Taking into account the available methods, and various complex factors that affect transport mechanisms, future studies should consider the combined application of various methods to study the physiological role of CreaT.

For instance, immunofluorescence microscopy approaches to CreaT expression and sub-cellular localisation could be combined with CreaT protein expression in muscle homogenate and high-energy phosphagens levels across different models (i.e. disuse, illness), which we were unable to do herein due to tissue availability. Based on our proposed hypothesis of the potential role of CreaT in short-term disused model, future studies should examine associations between disuse-induced muscle atrophy and alterations in Cr metabolism. This could be achieved in large-sample human trials or using CreaT KO animals. In addition, future studies should also determine the effect of prolonged disuse (>7-days) and subsequent recovery and re-loading on CreaT expression and sub-cellular localisation together with other comprehensive indices of Cr metabolism. Based on fibre-type specific CreaT content data generated in this thesis, future studies should examine the effect of manipulating training variables alters muscle Cr metabolism and CreaT activities in relation to muscle hypertrophy and strength accretion. Finally, studies are required to understand the effects of Cr supplementation interventions, along and combined with exercise training, on CreaT protein expression and sub-cellular localisation across the health-span.

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