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Musculoskeletal function and phenotyping in frail older patients having hip surgery following fracture.

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Abstract

A hip fracture is a catastrophic injury to an elderly individual with a significant impact individually, on the family, and for health care services. Despite optimisation of medical comorbidities, effective skilled anaesthesia, and early surgery, the patient important outcomes of mobility, independence, and mortality have not improved. With an aging population comes increasing frailty, and those who are frail have the worst outcomes. However, frailty in existing literature has been difficult to quantify. Multiple methods have been used to ascertain and classify frailty status, utilising two different schools of thought: cumulative deficits and phenotype models. Frailty is the ability for an individual to bounce back from illness or injury. It is not known why those who are frail have the worst outcomes and what biological processes occur which underpin these poor outcomes in these individuals.

This study describes and explains the effect of frailty on post-operative outcomes following hip fracture surgery. It evaluates local muscle gene expression using Vastus Lateralis muscle in both the non-injured and injured limb of a range of frail individuals to explain the underpinning biological process of frailty. Lastly it investigates the systemic inflammation secondary to trauma, and how this impacts mortality within this cohort.

The first experimental chapter examined the subjective and objective measures of muscle function and performance. It showed that there are significant differences between the extreme ends of the spectrum of frailty, those with a greater frailty had a poorer health related quality of life both at day 7 and day 90, mobility, institutionalisation, and mortality. Patients with severe frailty, clinical frailty scale score of 7, do not improve in mobility outcomes from day 7 post-surgery. Timed up and go test provides a useful binary measure to predict discharge home in patients who are admitted from their own home. The second experimental chapter identifies significant differentially expressed genes in the Vastus Lateralis muscles when comparing the fractured leg with the non-fractured leg. It also demonstrates no significant difference in gene expression in the non-fractured leg when stratifying for

frailty scores. However, in the fractured leg there is a significant difference in gene expression when comparing higher frailty states to lower frailty states. Key mechanisms of inhibition of apoptosis and cell death are reduced as frailty increases, and increased protein catabolism increases with increasing frailty. This highlights key mechanisms which may be potential targets to improve outcomes and prevent frailty. The third experimental chapter identifies the systemic inflammatory markers present at the time of hip fracture surgery and their relationship with 6-month mortality. Two biomarkers FGF-23 and IL-15RA are significantly raised and correlated with renal function which, are predictive of 6-month mortality. This highlights potential treatment targets for reducing inflammation and the role of optimisation of renal function in the peri and post operative management of hip fracture.

This study is uniquely placed amongst the existing literature for a myriad of reasons. It is the first study to investigate gene expression in the vastus lateralis muscle in the non-fractured leg and fractured leg of the same participants. It is also the first study to investigate gene expression in a range of frail patients identifying mechanisms which potentially underpin frailty and poor outcomes following hip fracture surgery. It is also the first study to investigate and evaluate a range of inflammatory markers using proteomic analysis and their relationship with 6-month mortality. The identification of novel biomarkers, cellular functions and objective assessments now paves the way for further research in determining novel therapeutic agents and rehabilitation strategies to not only further improve outcomes for hip fracture surgery but is translatable to reduce the impact of frailty on homeostatic reserve of elderly patients.

Declaration

All of the experimental data presented in this thesis were generated by me with the assistance of academic and technical staff at the David Greenfield Human Physiology Unit, and the Department of Academic Orthopaedics, Trauma and Sports Medicine, Queens Medical Centre, University of Nottingham; and Olink Proteomics Upsalla, Sweden.

For experiments in Chapter 5 participant recruitment, questionnaires, hand grip strength, were performed mainly by me. Ultrasound measurements and analysis were performed solely by me. Timed up and go test was performed by physiotherapy staff at Nottingham University Hospitals NHS trust, analysis performed by me. In Chapter 6 muscle biopsies were performed solely by me. Muscle mRNA expression and muscle protein expression analysis were carried out by Dr Joanne Mallinson and Dr Scott Cooper. Bioinformatics analysis was performed by Dr Mallinson.

For experiments in Chapter 7, venepuncture was performed by the anaesthetic team at Nottingham University Hospitals NHS trust, processing of samples and storage was performed by the staff in Academic Orthopaedics, Trauma, and Sports Medicine and me. Proteomic analysis was undertaken by Olink Proteomics Upsalla, Sweden.

I hereby declare that the work presented in this thesis, has not been submitted for any degree or diploma, at this or any other university, and that all the experiments, unless stated, were performed by me.

Signed:_____Adeel Ikram

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I would like to thank all members of staff at the David Greenfield Human Physiology Unit and Academic Orthopaedics, Trauma, and Sports Medicine with whom I have worked over the last four years. Thanks to Sara Brown, Joanne Mallinson, Scott Cooper, Tony Kelly, Waheed Ashraf, Jess Nightingale, Charlotte Bentley, and Professor Ana Valdes. My thanks also to Ben Marson, Simon Craxford, Abdullah Alqarni and Amy Zheng with whom I shared the PhD office. Our discussions have covered all aspects of research and life, providing a happy balance of fun and productivity.

I would like to thank my parents for being my inspiration to succeed, for pushing me to be the best I can be, keeping me grounded, and encouraging my detour into the scientific and academic world. I would also like to thank my brothers for giving me continuous moral support, especially when things became tough during the Covid-19 pandemic.

Finally, most of all, I would like to thank my partner Amna. The last four years have been a rollercoaster of emotion, compromises, hard work, teamwork, and perseverance. Amna has supported me through this PhD, my specialty training, and through the Covid-19 pandemic. She encouraged me to come in over the weekends to recruit patients, supported me to present my work at conferences, and helped lift my spirits when things became difficult. During this time, we have also had some of the best moments of our lives. We became new parents to our wonderful son Issa Imraan, and together it has been an absolute joy to watch him grow and learn. A wonderful partner and mother, she has been key in helping me to achieve this PhD. Without Amna and Issa's love, understanding, unconditional and unwavering support, none of this would have been possible.

I dedicate my thesis to my late grandfather Abdul Ghani Ibrahim whom I miss dearly. A pious man and incredibly humble man, he valued the acquisition of knowledge, and education. From the outset he told me to become a 'bone doctor'. His last lesson for me was to always remember 'it's not what you do for yourself, it's what you do for others that matter. Sadaqah Jariyah.'

Publications arising from thesis

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Can the Clinical Frailty Scale on admission predict 30-day survival, postoperative complications, and institutionalization in patients with fragility hip fracture? A cohort study of 1,255 patients. Bone Joint J. 2022 Aug;104-B(8):980-986.

Ikram A, Norrish A, Ollivere L, Nightingale J, Valdes A, Ollivere BJ
Has a change in established care pathways during the first wave of the COVID-19 pandemic led to an excess death rate in the fragility fracture population? A longitudinal cohort study of 1846 patients BMJ Open 2022;12:e058526. doi: 10.1136/bmjopen-2021-058526.

Valdes A, **Ikram A**, Taylor L, Zheng A, Kouraki A, Kelly A, Ashraf W, Vijay A, Miller S, Nightingale J, Selby N, Ollivere B
Preoperative inflammatory biomarkers reveal renal involvement in postsurgical mortality in hip fracture patients: an exploratory study Front Immunol.2004 Jun 10:151372079.

Presentations arising from thesis

International Podium

Orthopaedic Trauma Association	2024
European Federation of National Associations of Orthopaedics & Traumatology	2024
European Federation of National Associations of Orthopaedics & Traumatology	2024
Orthopaedic Trauma Association	2023
Orthopaedic Trauma Association	2022
Orthopaedic Trauma Association **(Best International Paper)**	2021
Orthopaedic Trauma Association	2021
European Federation of National Associations of Orthopaedics & Traumatology	2020
European Federation of National Associations of Orthopaedics & Traumatology	2020

National Podium unless stated

British Hip Society **(Top 20 Poster)**	2024
British Hip Society (Poster)	2024
Royal Society of Medicine Orthopaedics Presidents Prize **(2nd Prize)**	2022
Royal Society of Medicine Trauma Symposium	2022

Regional

East Midlands North Orthopaedic Registrar Day **(Best Academic Paper)**	2024
Malkin Orthopaedic Research Meeting **(3rd Prize)**	2023
East Midlands Orthopaedic Research Meeting **(1st Prize)**	2023
Malkin Orthopaedic Research Meeting **(1st Prize)**	2022
East Midlands Orthopaedic Research Meeting	2021
Malkin Orthopaedic Research Meeting	2021
East Midlands Orthopaedic Research Meeting **(1st Prize)**	2020
Malkin Orthopaedic Research Meeting	2020

Covid-19 Pandemic Mitigation Statement

This study was commenced during the Sars-Cov-2 global pandemic. For the first year of the PhD, the study could not be commenced as no new non-covid studies could be opened. This was due to all research endeavours being focussed on investigating the impact of the Sars-Cov-2 virus, and to facilitate the production of vaccinations and testing. Laboratory equipment, research staff, time, and finances were seconded to facilitate this research. Members of the research team including clinicians, research nurses and allied staff had been redeployed to frontline services. To facilitate the opening of the FEMUR study, an agreement was in place where, the majority of this study was completed by the clinical research fellow. As a result, some aspects of the study were not able to be completed. Specifically, the intramyocellular lipid content of the muscle samples. For this to occur it required an additional research member to process the sample soon after it was taken in theatre which was not possible due to the shortage of research staff. Secondly the urinalysis of the D3 creatine given to a subgroup of the patients was not completed as part of this thesis due to the increased workload and use of laboratory equipment to facilitate the research and production of the Covid-19 vaccines. But the samples were collected and stored for analysis to be undertaken at a later point. Thirdly the bone sample analysis has been undertaken but not included in this thesis, this was due to the remaining time constraints to meet the submission deadline.

During the first year of the PhD, I was part of the Pandemic Tracking of Health Care Workers (PANTHER) research team. Research was undertaken into the effect of the Covid-19 pandemic on healthcare workers. This resulted in publications in high impact science journals and informed vaccination practice in the United Kingdom following presentation to the Scientific Advisory Group for Emergencies (SAGE) committee during the pandemic.(1, 2)

The Covid-19 pandemic also had a significant impact on me personally. My partner and I undertook significant risks to our personal and mental health. My partner Amna worked on the front line in intensive care and respiratory units looking after those directly admitted with Covid-19, and I when undertaking trauma on calls and operating lists as a specialty trauma and orthopaedic registrar. We cross covered colleagues who fell ill when the need arose. Many of our days were spent worrying about the possibility of being infected, admitted to hospital and facing death without seeing our families. Fear of our loved ones also being infected prior to the development and rollout of the national vaccine also had an impact on our mental health.

A significant number of my personal family members passed away during the pandemic due to infection of the virus. The disease was indiscriminate in its approach affecting the young and old. We were unable to grieve with and provide comfort to our families or attend the funerals. Other family members were admitted to hospital and intensive care with no possibility for visitors due to infection risk.

As a couple we faced significant isolation from family and friends due to the national lockdown. We were also in the difficult place of being healthcare workers in rented accommodation and asked to vacate our property due to our risk profile. As a result, when trying to purchase our first home we faced significant delays due to the pandemic. This resulted in us having no fixed abode for two weeks with all our belongings in storage adding to an already incredibly stressful time.

Despite things returning to normality, we are unaware of the long-term implications of the pandemic and multiple episodes of infection of Covid-19 we sustained. Only time will tell how Covid-19 has affected us long-term physically and mentally.

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1 Introduction

1.1 Chapter Overview

This chapter aims to contextualise the research question. It discusses the importance of hip fracture in the elderly, its management, how outcomes are measured, and how frailty impacts recovery. This chapter also highlights how sarcopenia the physical component of frailty plays a role, and the difficulties clinicians face in trying to improve outcomes in an aging patient population.

1.2 Hip fracture in the elderly.

1.2.1 The magnitude of the problem.

1.2.1.1 Incidence of hip fracture in the UK and the world.

In 2050, the global annual incidence of patients presenting to hospital with a hip fracture is expected to be 6.26 million, a rise from 1.62 million in 1990.(3) The incidence in the United Kingdom (UK) is approximately 76,000 per year, with the majority occurring in those aged above 70. This injury has a high mortality rate; 8.3% of patients die within a month and 30% within a year.(4) Many patients develop complications from their injury, age, and frailty related comorbidities. This catastrophic injury causes patients to lose their independence and confidence in their ability to care for themselves. The level of frailty exhibited by patients with hip fractures places a significant financial burden on health care systems for the management of these patients. The 2019 annual report of the National Hip Fracture Database (NHFD) indicated that 1% of the entire UK government budget, £1 billion, is spent on the medical and social care of patients with this injury.(5)

1.2.1.2 Mortality & morbidity of hip fracture

Patients with hip fractures frequently have substantial medical comorbidities, and the combination of a fall and fracture frequently reveals underlying illness. Local and systemic infection, venous thromboembolism (deep vein thrombosis and pulmonary embolism), death, muscle atrophy due to immobility, delirium and cognitive impairment, loss of independence, and institutionalisation are all possible

complications. Data from the NHFD 2021 annual report showed the 30-day mortality rate rose from 6.5% in 2019, to 8.3% in 2021. During the Covid-19 pandemic, 30-day mortality was also three times higher for those infected with Covid-19.(6) This highlights how dynamic and sensitive the outcomes for these patients can be, in response to changes in established care pathways, concurrent conditions, and changes to government legislation. This is an important factor to consider particular to this study which was undertaken during the Sars-Cov-2 global pandemic and will be addressed further in this thesis.

1.2.1.3 Impact on social care and patients' independence

Patients who have sustained a hip fracture experience significantly worse mobility, independence, health, quality of life, and higher rates of institutionalisation than aged-matched controls. The main period of recovery for these patients has been shown to occur within the first six months after sustaining the fracture. A systematic review has shown that 40-60% recovered pre-fracture level of mobility and ability to perform activities of daily living.(7) However, the data represents mobility in survivors and therefore it may actually indicate longer survival in patients with better pre-injury mobility and lower frailty. Japanese and Swedish studies, have shown the proportion of patients who have survived four months is similar, to those at one to two years.(8-10)

A year following hip fracture, patients are less likely to drive, leave the home, work, or volunteer. These patients report significant depressive symptoms. They experience significant functional limitations and are unable to engage in their preferred activity for up to two years. Those with poorer health have higher mortality rates and are consequently more likely lost to follow-up. These estimates show the lower limits of true hip fracture related decline in psychosocial well-being. Existing studies have not shown the contribution of postoperative rehabilitation strategies which may have also affected these figures.(11)

Using data from the World Hip Trauma Evaluation (WHiTE) cohort, a feasibility study assessed the cost of formal and informal care, home adaptations, and physiotherapy

for elderly patients with hip fracture. The data in WHiTE are representative of the hip fracture population in the UK. Estimated mean costs of formal and informal care, home adaptations, and physiotherapy were £2,843 (SD: 5,467), £6,613 (SD: 15,146), £706 (SD: 1,706), and £9 (SD: 33) for 3524 patients in the four months following injury respectively. Informal care is a significant source of expenditure after a hip fracture, but the interpretation of informal care requirements varies considerably.(12)

1.2.2 The inflammatory burden of trauma and the surgical management of hip fractures.

1.2.2.1 Inflammatory burden of trauma

The relationship between inflammation and mortality in patients who have sustained severe trauma is complex and multifactorial. Excessive systemic inflammation is known as the systemic inflammatory response syndrome (SIRS). This condition can lead to sepsis, organ dysfunction, multiple organ failure, coagulopathy, haemorrhage, and death.(13) Prolonged activation of the immune system can lead to immunosuppression increasing the risk of secondary infection and complications. The management of inflammation is important to modulate its response, this helps to mitigate the harmful effects of excessive inflammation and improve outcomes.

Surgery increases the likelihood of a systemic inflammatory response and in combination with trauma the effect may be compounded.(14) This has also been shown specifically in patients following hip fracture surgery, where there is a greater incidence of post operative SIRS and sepsis.(15) Although age has been linked to a prolonged inflammatory response,(16) recent studies have demonstrated patients who are frail have an enhanced inflammatory response which is associated with a higher mortality rate.(17) However, there is a paucity in data in those patients as frail as those who have sustained a hip fracture.

With an aging population, the number of elderly patients who sustain trauma has increased. Despite the nature or severity of their injury and considering comorbidities, the prognosis for the elderly following trauma is significantly worse.(18, 19) The

association between age and injury outcome may be confounded by factors that contribute to the risk of injury in the first instance, even following risk adjustment for known confounders. However, residual confounding is unlikely to explain the magnitude of the relationship between age and mortality, which varies three to four fold between the second and ninth decades of life.(20, 21) A biological definition of ageing is the progressive deterioration of an organism's resistance to stress as it ages, a fall in the stress range over which the body can maintain homeostasis. Numerous hypotheses have been proposed despite the fact that the precise mechanisms linking age to the capacity to endure the stress of trauma have not been elucidated.(22) It is known that elderly individuals have a baseline state of low-grade inflammation, known as inflammaging which may be a contributing factor.(23)

Age increases the likelihood of a sustained inflammatory response for a given severity of trauma. Despite an increase in non-specific immunity, SIRS is paradoxically accompanied by a decrease in the body's ability to erect a defence against invading pathogens. Invading microorganisms stimulate immune cells through pathogen-associated molecular patterns, thereby increasing susceptibility to infection and sepsis. SIRS results in inflammation and immunoparesis, which leads to sepsis with additional inflammation and the possibility of multiorgan failure (Figure 1.1). The inflammatory response also includes rapid activation of the complement system; initial activation is followed by consumption and an imbalance in the complement cascade's components, which is one of a number of factors that decreases the body's ability to defend itself against microorganisms.(16)

The SIRS response may be exaggerated in elderly individuals following injury, possibly because their bodies have been primed by elevated baseline levels of cytokines or because they have a diminished capacity to produce anti-inflammatory cytokines such as interleukin. Immunosenescence is the age-related decline of both the non-specific (innate) and specific (adaptive) immune pathways, which diminishes the ability to respond to pathogens.(23) A raised cortisol: dehydroepiandrosterone sulfate (DHEAS) ratio is seen in all patients after trauma. The age-related decline of Dehydroepiandrosterone (DHEA) and DHEAS may exacerbate this increase and

contribute to immunoparesis in elderly trauma patients.(24) In addition to old age, large retrospective studies have identified male sex as a significant risk factor for pneumonia and sepsis following severe trauma, despite the lack of consensus.(25) This observation may be attributable to the modulation of pro-inflammatory and anti-inflammatory pathways by oestrogen.(26)

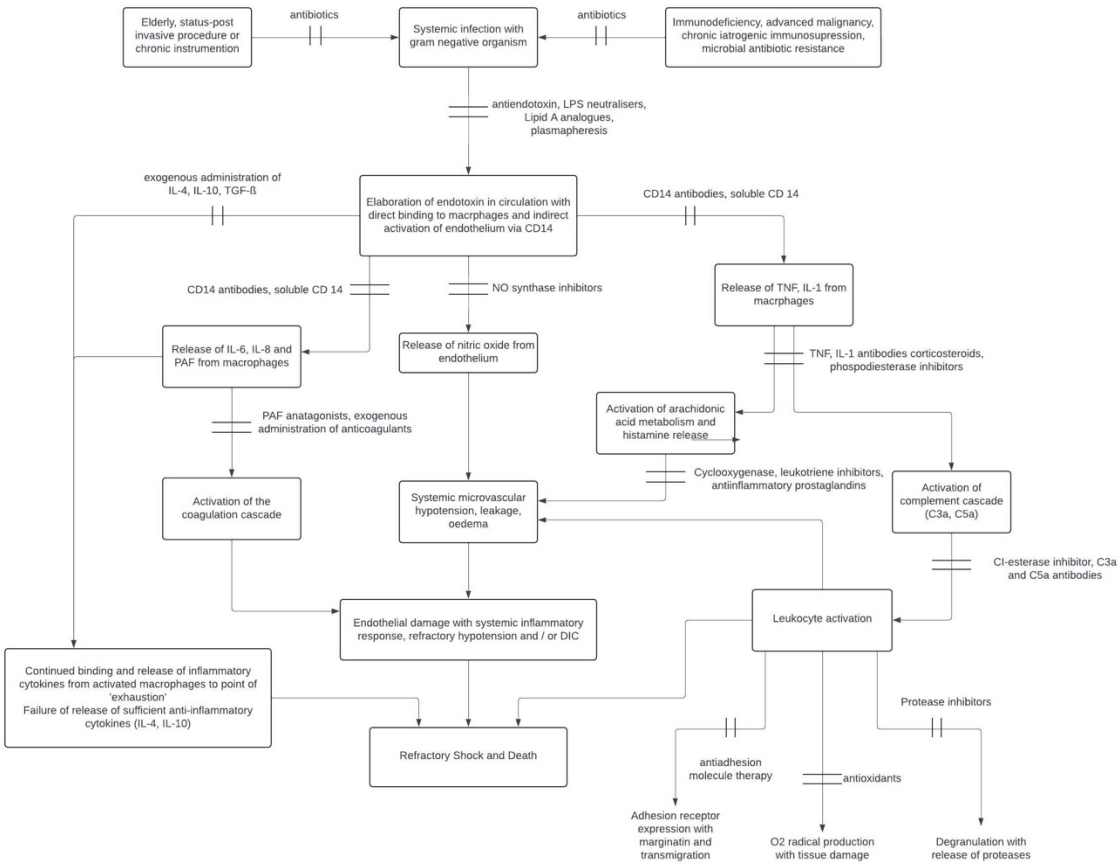


Figure 1.1 Adapted from Evolving strategies in the treatment of sepsis and systemic inflammatory response syndrome (SIRS) by K Horn 1998 QJM an international journal of medicine. The inflammatory cascade in septic shock. Illustrated are the points where some experimental approaches may prove therapeutic. These points are represented by double lines.(27)

SIRS is an inflammatory response to blood loss and tissue damage linked to traumatic injuries, which occurs after thirty minutes. There is a release of endogenous factors damage-associated molecular patterns (DAMPs) from neutrophils and necrotic cells.(28, 29) DAMPs directly act on DAMP receptors on the surface of immune cells (neutrophils and monocytes) to accelerate the production of complement activators C3a and C5a.(30, 31) SIRS is also triggered by other inflammatory mediators such as interleukin. The pathways leading to SIRS are the targets of numerous anti-multiorgan failure therapies. With SIRS there is a compensatory anti-inflammatory response characterised by anti-inflammatory cytokines and cytokine agonists.(32) The ratio of pro-inflammatory to anti-inflammatory factors results in either a return to base line levels or chronic inflammation, immunosuppression and catabolism syndrome which has an increased risk of multi-organ dysfunction and sepsis.(33)

Several inflammatory markers have been associated with the fragility hip fracture population predominantly with incidence of hip fracture.(34) One study demonstrated Interleukin-6 and Tumour necrosis factor alpha being elevated in the first few days following surgery. These markers have showed no relationship with 2-year mortality, however as the markers were obtained following surgery it is difficult to ascertain if the rise was associated with the hip fracture or surgery itself.(35) Another recent study demonstrated elevated plasma neopterin levels a molecule released by interferon gamma activated macrophages was predictive of survival after hip fracture.(36) One study has examined the possible association between 30-day mortality following hip fracture and known biochemical markers of inflammation. They demonstrated increased mortality in patients with elevated levels of soluble urokinase plasminogen activator receptor (suPAR), C-reactive protein (CRP) and ferritin and low levels of transferrin on admission. The excess inflammatory response is likely caused by muscle damage associated with hip fracture. However, this study was limited due to a lack of clinical information about possible causes of or contributors of elevated inflammatory response, its retrospective design and no reliable information on cause of death.(37) The effect of hip fracture and surgery induces a systemic inflammatory response. This has been shown in rat models to lead to lung injury from increased susceptibility to infection.(38) Most of the existing

studies have looked at only a few inflammatory markers within a cohort of patients. Given the multiple inflammatory mediators and pathways as part of the inflammatory response of trauma and surgery this represents a challenge, firstly to identify a range of biomarkers within a cohort of patients and secondly how these relate to outcomes not only mortality but complications post-surgery and recovery. The ideal scenario would be to screen a range of markers within a cohort of patients with hip fracture but prior to surgery and observe the outcomes of these patients. Common inflammatory cytokines with their function and cell origin are showed in Table 1.1.

Table 1.1: Common cytokines and their cell sources, with examples of their principal function in inflammation. Abbreviations: IFN, interferon; IL, interleukin; NK, natural killer; NF-κB, nuclear factor-kappaB; Th, T helper; TGF, transforming growth factor; TNF, tumour necrosis factor.

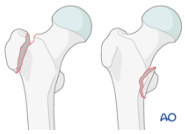
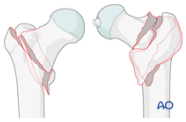
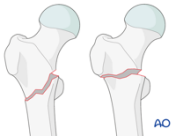
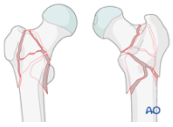
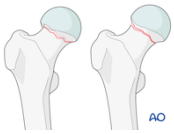
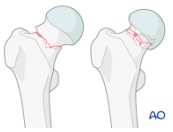
Cytokine and Cell source	**Key functions inflammation**
IL-1: Macrophages, Endothelial cells	Synthesis of acute phase proteins by hepatocytes; Local and systemic inflammation
IL-2: Activated T cells, Th1 cells	Proliferation of T cells, B Cells; Proliferation and activation of NK cells
IL-6: Macrophages, Th2 cells, Endothelial cells, Adipocytes, Myocytes, Osteoblasts	Synthesis of acute phase proteins by hepatocytes; Proliferation of B cells; Reduce IL-1 and TNF production; Activate immune cells, osteoclasts, endothelial cells: Hypothalamic Pituitary Axis – fever and hormone release
IL-10: Macrophages, Th2 cells	Resolution of inflammation; Inhibition of Th1 inflammatory cytokine synthesis; Inhibition of activated macrophages and dendritic cells
IL-12: Macrophages, Dendritic cells	Promotion of Th1 differentiation; Stimulation of IFN-ɣ production by T cells, NK cells
TNF-α: Macrophages, T cells, NK cells, Lymphoid cells, Endothelial cells, Adipocytes, Cardiac myocytes, Fibroblasts, Neuronal cells	Synthesis of acute phase proteins by hepatocytes: Recruitment and activation of neutrophils and monocytes at site of infection; Stimulation of CRP release from liver; Activation of NF-κB pathway; Induction of insulin resistance
TGF-β: Macrophages, T Cells	Resolution of inflammation; Limit production of IL-2, IFN-ɣ, and TNF; Inhibit of proliferation / activation of B cells, T cells, macrophages
IFN-ɣ: Th1 cells, NK cells	Activate of macrophages; Suppression of Th2 cell activity: Promotion of leukocyte migration

1.2.2.2 Classification of hip fractures

Fractures of the hip can be broadly categorised according to age (young or old), mechanism (low or high energy), and anatomy (intracapsular or extracapsular). Each classification type provides information and modifies surgical patient management. Young hip fracture can be defined as aged under 50 and is usually the result of high energy trauma.(39) Other studies have identified that the risk of fragility hip fracture increases from the age of 60-65 which can be deemed as old.(40, 41) Patients between this age range are difficult to categorise and provide much debate on their management. In this study as we are investigating the effect of frailty, we have used the age cut-off as 65 and above. This is because the Clinical Frailty Scale Score which is used to assess patients for frailty at admission is only validated in those 65 years and older.(42) Anatomical classification is equally as important. Hip fractures are classified anatomically based on their relationship to the femoral head's capsular attachment and terminal vascularisation. The two forms are intracapsular and extracapsular, with intertrochanteric and subtrochanteric constituting the latter.

Intracapsular hip fractures are fractures of the femoral neck because they occur within the hip joint but exclude the femoral head. These fractures are further categorised as either non-displaced or displaced. Disruption of blood flow to the femoral head in displaced intracapsular fractures, carry a high risk for avascular necrosis. Fractures that are not displaced are associated with impaction or adduction. Extracapsular hip fractures are located distal to the hip joint, but within 5cm of the inferior most aspect of the lesser trochanter. Fractures of the intertrochanteric region extend distally from the femoral neck to the lesser trochanter. They are located between the main abductors and flexors of the hip. Subtrochanteric fractures are located between the inferior border of the lesser trochanter and the 5cm of bone that extends inferiorly from this border. For clinical purposes, classifications as simple as those described above are utilised. In contrast, the Müller Arbeitsgemeinschaft für Osteosynthesefragen (AO) classification is used in research because it is one of the few complete, exhaustive, and validated fracture classification systems currently in use (Table 1.2.)(43)

Table 1.2: AO classification of hip fracture, adapted from AO Surgery reference (44)

	Description	Classification
	Pertrochanteric Simple	
	Isolated single fracture of the greater and lesser trochanter	31A1.1
	Pertrochanteric, 2-part	31A1.2
	Intact lateral wall	
	Pertrochanteric with posteromedial involvement	31A1.3
	Intact lateral wall	
	Pertrochanteric multifragmentory	31A2
	Incompetent lateral wall	
	Intertrochanteric (reverse oblique)	31A3
	Simple oblique	31A3.1
	Simple transverse	31A3.2
	Wedge or multifragmentory	31A3.3
	Subcapital femoral neck	
	With valgus impaction	31B1.1
	With little or no displacement	31B1.2
	Displaced	31B1.3
	Transcervical femoral neck	
	Simple	31B2.1
	Multifragmentory	31B2.2
	Shear	31B2.3
	Basicervical	31B3

1.2.2.3 Surgical management of hip fractures

The National Institute for Clinical Excellence (NICE) has guidelines for the multidisciplinary management of hip fractures. It consists of early surgery, skilled anaesthesia, and an orthogeriatric care and rehabilitation package. In most cases, displaced intracapsular hip fractures are treated surgically with a hemi-arthroplasty or a total hip replacement (THR), with the latter procedure offered to those who are medically more robust and can walk with at least one cane.(41) Non-displaced intracapsular hip fractures can be treated with either fixation via cannulated screw fixation or 2-hole dynamic hip screw, or hemiarthroplasty. Hip fixation is reserved for patients who meet specific demographic and clinical criteria. Fractures of the intertrochanteric hip region are treated with a dynamic hip screw (DHS). Subtrochanteric hip fractures, reverse oblique intertrochanteric fractures, and fractures with medial calcar failure are treated with an intramedullary nail.(41) As previously stated, the difference between surgical fixation and replacement is founded on the blood supply of the femoral head. Patients with an intracapsular hip fracture are more likely to develop avascular necrosis, which causes the femoral head to collapse, resulting in pain and difficulty walking. Therefore, arthroplasty, in which the femoral head is replaced, is offered. With extracapsular hip fractures, the blood supply is preserved, so these fractures can be fixed. The choice of implant is then determined by the biomechanics of the fracture pattern to maintain fracture reduction and stability until the bone heals. The primary objective of treatment for elderly patients is to alleviate pain and permit early mobilisation. This is done to prevent the more severe complications of immobility, such as respiratory and urinary tract infections, venous thromboembolism, pressure ulcers, institutionalisation and dependence, and mortality. On the basis of a patient's age, pre-existing conditions, and physiological parameters at admission, scoring systems have been developed to predict mortality.(40) All hip fracture information is collated and centralised on the NHFD. Where epidemiological data and outcomes can be monitored to enhance national patient care.(4)

Along with NICE and the NHFD, the British Orthopaedic Association (BOA) have standards of care for patients with fragility fractures and patients sustaining major

trauma with a clinical frailty scale score of 5 or more. Specific to the hip fracture cohort they advise all patients should receive a comprehensive geriatric assessment within 72-hours of injury, ceiling of treatment including transfer and escalation and appropriateness of cardiopulmonary resuscitation should be discussed with the treating teams and documented. All patients should have a falls assessment and referred to the falls prevention service. Delirium should be regularly monitored. Surgery should allow the patient to fully weight bear and patients should be reviewed day-1 following surgery. Rehabilitation strategies and pathways should be used for all patients, and finally each hospital should submit data to national databases to monitor performance against national benchmarks.(45)

1.2.3 The hip joint and the implications of ageing on bone and muscle.

1.2.3.1 Hip joint anatomy and blood supply.

The hip is a synovial ball and socket joint, with the spherical head of the femur and cup of the acetabulum providing the primary stability. The surrounding fibrous capsule, ligaments, and muscles traversing the joint provide secondary stability. (46) The medial femoral circumflex artery and inferior gluteal artery are the two major arteries that supply the femoral head. Both are essential for comprehending the surgical approach and extent of injury following a fracture, with a focus on avascular necrosis.(46)

1.2.3.2 Aging in bone

Bones undergo a lifelong process of remodelling according to Wolff's law. (47) Mature tissue is removed, and new bone is formed. Homeostasis is regulated closely with a balance between resorption and formation to maintain skeletal integrity. With increasing age there is reduced mineral content becoming prone to osteoporosis, a condition where bone becomes more brittle, fragile, and prone to fracture all secondary to reduced density. In ageing there is a change in balance of resorption and formation with the latter reduced. This occurs because of an inactive lifestyle, hormonal changes and loss of calcium and minerals within bone. Within the trabecular bone, the age-related loss in men is predominantly due to thinning of the individual

struts. In women there is a decrease in connectivity, this is more apparent following the hormonal changes of the menopause. With age bones become stiffer and the cross-sectional area decreases.(48) As the load per unit area increases and bone deformation occurs in fragile bone, once past a threshold this leads to fracture.

The two main age-related conditions which affect hip anatomy include osteoarthritis and osteoporosis. Osteoarthritis is highly associated with advancing age leading to a loss of joint space, pain and stiffness and it increases the risk for hip fractures.(49, 50) After 60 years there is an increased risk of osteoporosis which results in a decrease in bone mineral density which increases the risk of hip fracture.(51)

Other implications of advancing age such as ischaemic heart disease, gait disturbances, polypharmacy, dementia, muscle weakness, balance problems, and other chronic diseases can further increase the risk of falls and thus fractures.(52) The metabolic changes which occur in the bone due to the process involved in aging result in the bone fracturing under a lower force being applied through it. A fall from a standing height or low energy trauma is often described as a fragility fracture.(53)

1.2.3.3 Aging in muscle

Progressive changes in skeletal muscle mass, metabolism, and functional capacity are associated with a decline in independence and an increase in mortality with ageing. (54) Busse, first introduced the concept of primary and secondary degeneration in muscles. Primary ageing refers to the intrinsic time-related biological processes that are unrelated to stress, trauma, or disease, whereas secondary ageing refers to physiological decline caused by environmental and lifestyle factors like diet and exercise. (55) Therefore, secondary ageing may be partially reversible or preventable by modifying environmental, dietary, and exercise factors.

Loss of muscle mass (previously termed sarcopenia) and diminished strength and quality (dynapenia) are consequences of ageing. There is no consensus in the scientific literature regarding the rate of decline of muscle mass; however, cross-sectional studies indicate differences between young (18-45 years) and elderly (65-90 years) of

8% to 45%. However, these studies assume that the elderly and the young are equally matched and do not take intergenerational differences into consideration.

Longitudinal studies have revealed that the lean muscle mass of the legs decreases between 0.7% and 0.8% per year, with the decline being greater in those aged 70 and older. (56) This loss is linear and accelerated after age 60 in later life. (57) The rate of muscle mass loss is double in the lower extremities when compared to the upper extremities, as indicated by a number of studies. (58) Studies investigating bed rest reveal that the elderly (mean age of 67) lose more lean tissue after 10 days of immobilisation than younger individuals do after 28 days of immobilisation. (59) According to one study, young volunteers lost more quadriceps muscle after 14 days of immobilisation than older volunteers (mean age of 67 years).(60)

Balance between muscle protein synthesis (MPS) and muscle protein breakdown (MPB) regulates muscle mass. This equilibrium is influenced by diurnal variation, dietary intake(61) and physical activity.(62) The anabolic effect of nutrition is driven by amino acids from dietary proteins incorporated into skeletal muscle. Anabolic resistance leads to chronic muscle mass loss via inability to stimulate MPS or inhibit MPB following feeding or exercise. Cuthbert et al compared the rate of MPS in 44 healthy young and older men in response to essential amino acids they discovered that the elderly demonstrated a blunted myofibrillar protein fractional synthetic rate in response to essential amino acids in comparison to the young, highlighting the inability of aged muscle to detect a nutrient signal.(63) Similarly, Kumar et al demonstrated a blunted MPS response 1-2 hours after resistance exercise training in older men when compared with young men.(62) Brook et al suggested age-related failure of muscle hypertrophy occurring earlier in life than previously expected with only the young 18-28 years showing a response.(64) Studies have also shown a loss of quadriceps cross sectional area may be attributed to loss of mitochondria adenosine triphosphate (ATP) capacity, which impact negatively on muscle metabolic resilience and the ability of skeletal muscle to respond to and recover from physiological stress of contraction.(65) Muscle metabolic resilience can also be affected by reduced intrinsic mitochondrial function and ATP production per mitochondria through

reactive oxygen species and defected autophagy.(66) Collective physiological aging is associated with muscle loss and reduced metabolism capacity and reduced resilience, which leads to reduced functional ability and further age-related decline.

The magnitude of strength loss is greater than mass loss which has been demonstrated in studies by observing hand grip strength(67) and lower limb strength.(68) Delmonico et al studied 1678 older individuals with follow up over 5 years showed knee extensor torque reduced 2-5 times more than the cross-sectional area of the thigh. Fontera et al showed a 20-30% decline in knee flexion and extension with mean 16% loss in quads cross sectional surface area in older individuals over 12 years follow up. However, there were significant differences in habitual activity levels from the 1st to 12th years, loss occurred due to aging but some of the loss may have been due to the reduced habitual activity level due to age.(69)

Power, the product of force and velocity, has been reported to decline more rapidly than strength with age, especially in older age when mobility is limited.(70) A preferential loss of fast twitch muscle fibres leads to, low muscle mass which in turn is associated with a higher mortality. This is independent of fat mass and cardiovascular risk factors(54)

In muscle the three main types of muscle fibres are type 1 (slow twitch), and type 2a and 2x (fast twitch). Each fibre has different biochemical and physiological characteristics. A loss of predominately type 2, mostly 2x fibres occurs with aging.(69, 71, 72) Some have argued that this occurs in part secondary to age related remodelling of motor units resulting in denervation of type 2 fibres and subsequent collateral formation of type 1 fibres.(71) Others have suggested that there are fibre type differences between the old and young individuals.(73)

With aging there is a decline in motor unit numbers when comparing older people with young, and with aging there is an increase in size of surviving motor units via reinnervation of denervated fibres.(74). Healthy older men can reinnervate large numbers of muscle fibres to compensate for declining motor unit numbers, failure to

do so leads to sarcopenia. This ability could explain the difference between pre-sarcopenia and sarcopenia where there is a lack of compensatory reinnervation of muscle fibres from the former to the latter. The role of exercise is to preserve motor unit number in aging to improve reinnervation of denervated fibres is unclear. In patients with hip fracture this is of paramount importance as not only are the patients bed ridden until their surgery but many of them have reduced mobility because of undergoing surgery, its complications, complications of patient's co-morbidities and pain.

1.3 Measuring outcomes in patients with fragility hip fracture.

Hip fracture is a complex diagnosis with multiple potential outcome domains. National performance is benchmarked against several hard outcomes including mortality, length of stay and surgical complications. The National Hip Fracture Database (NHFD) is a national clinical audit conducted by the NHS to track the outcomes of hip fracture patients. As part of the Falls and Fragility Fracture Audit Programme (FFFAP), it is commissioned by the Health Quality Improvement Partnership and managed by the Royal College of Physicians. (4) It was originally created by the British Orthopaedic Association and the British Geriatrics Society to improve the quality and cost-effectiveness of hip fracture therapy. It employs a number of performance indicators to track improvements or deficiencies in hip fracture care, including but not limited to admission to a specialist ward, prompt orthogeriatric assessment and surgery, choice of anaesthesia, assessments of delirium, nutrition, cognition, falls, physiotherapy, type of fracture mobility, and 30-day mortality.(4)

Patients however in general prefer other outcome measures. In a comprehensive review of appropriateness of various outcome measures Griffin et al (75) identified a number of domains that should be included in the core outcomes of any hip fracture study. Their five domains were mortality, pain, activities of daily living, mobility and health related quality of life (HRQL) They concluded their work with suggesting that single-item measures of mortality and mobility (indoor/outdoor walking status) and

generic multi-item measure of HRQL – the EuroQol EQ-5D should be included in all hip fracture studies in addition to study specific outcomes.(75)

1.3.1 Survival

1.3.1.1 Current survival rates

Survival is one of the core outcomes identified as important to patients in the core outcome set work.(75) Currently in the UK the overall 30-day mortality is 8.3%; and 1 year survival 30%.(4) Survival is related to a number of confounding factors and validated predictors of survival are useful tools to understand the underlying risks for adverse outcome.

1.3.1.2 The Nottingham Hip Fracture Score

The Nottingham Hip Fracture Score (NHFS) was established in 2007 as a result of a cohort study involving 4967 patients.(76) A group of variables were independent predictors of 30-day mortality. These variables included age, gender, number of comorbidities, mini-mental test score, admission haemoglobin concentration, residing in an institution, and the presence of malignancy. The variables were incorporated into a risk score whose area under the receiver operating characteristic curve was determined to be 0.79. In 2011, it was considered a reliable predictor of 30-day mortality following validation of the score in three UK hip fracture centres.(40) A one-year validation of the risk score has also been conducted using data from local hospital statistics and the office of national statistics.(77) Nationally the NHFS is widely used as a predictor of 30-day and 1 year mortality. Other scoring systems are available and have been used in hip fracture care.

In 1991, the Physiological and Operative Severity Score of enumerating mortality and morbidity (POSSUM) was developed to assess the prognosis of patients undergoing general surgery.(78) Its improvement led to the development of the P-POSSUM. It consists of two major components: a physiological scoring system and a surgical scoring system. There is evidence of its use in orthopaedic surgery.(79) A recent systematic review found that both can accurately predict morbidity and mortality in

patients with hip fracture, but orthopaedic surgery-specific enhancements are needed.(80) A meta-analysis revealed that this score tends to overpredict in low-risk populations and at extreme ages.(81) The hip fracture cohort is distinct from the general surgical patients for whom the score was originally devised. Due to advancements in hip fracture surgery and treatment, it is recommended that, when using this scoring system, unit scores should be expressed as an observed: expected mortality rate, necessitating the accumulation of data over time.

1.3.2 Post operative complications.

The WHiTE study recorded the incidence of complications in the first 120 days following hip fracture. An analysis of 8673 consecutive patients revealed the following leading complications; wound infection, dislocation, failure of fixation, peri-prosthetic fracture, revision surgery, blood loss requiring transfusion, chest infection, urinary tract infection, deep vein thrombosis (DVT) /pulmonary embolus (PE), cerebrovascular accident, acute coronary syndrome/myocardial infarction and acute kidney injury.(82) These complications place a significant increased burden on frail patients and may further impair their ability to bounce back following a hip fracture.

1.3.3 Patient reported outcomes.

A patient reported outcome (PRO) is directly reported by the patient without interpretation. It can reflect their health, quality of life, functional status and is associated with health care or treatment. It is important to understand the patient's perspective as it provides a more holistic interpretation and comprehensive evaluation of the benefits of treatment alongside the traditional survival, disease, and psychological outcomes. Effectiveness of intervention has many dimensions including the clinical effectiveness of the intervention and the benefit to the patient using a variety of instruments to measure functional status, service satisfaction and quality of life. The patient experience plays an important role in clinical research and continues to increase in recognition due to a patient-centred approach. Therefore, this comprehensive assessment is necessary to evaluate the impact of injury or diseases and treatment. PRO can be used as a primary or secondary outcome, while a patient

reported outcome measure (PROM) is a measurement of the PRO, such as quality of life.

PROMs are tools or instruments to measure PROs, which are often self-completed questionnaires and measure functional status, health related quality of life, symptom and symptom burden, personal experience of care, and health related behaviours such as anxiety or depression. They can be broad based or disease specific. Broad-based PROMs examine a variety of different conditions and allow comparison across conditions and allow evaluation of new methods of providing care and service delivery and cost effectiveness and cost utility analysis. Disease specific PROMs are designed to identify specific symptoms and their impact on the function of the specific conditions. They demonstrate greater face validity and credibility than generic PROMs, but these comparisons cannot be made across a variety of conditions. Clinical studies use a combination of broad based and disease specific PROMs. NICE recommends using PROMs in comparative studies to provide evidence of a service's efficacy. There are numerous PROMs, some of which have been widely implemented. However, it can be challenging to select an appropriate PROM, and patient questionnaires can be burdensome. Ideally, disease-specific measures must be utilised; however, if no disease-specific measures exist, an alternative may be necessary.

PROMs have several methodological issues which must be considered when used in clinical research including validity, sensitivity, reliability, generalisability, and feasibility. One issue involves the identification and selection of valid, sensitive PROMs as a variety of PROMs have stemmed from clinical research. The validity of PROMs is based upon whether they measure what they intended to do a term defined as construct validity. The content validity is equally important when selecting a PROM and is established through analysis of the instruments content and concept that the test is designed to measure. Therefore, a precise and reliable instrument must be valid and responsive and or sensitive to change when evaluating treatment differences between groups if they exist. The choice of PROM to be selected is based upon professional judgement versus conceptual modelling. Conceptual modelling in the

context of selecting a PROM involves creating a framework which defines key concepts, constructs, and relationships relevant to the study. Through this, a solid foundation can be laid to ensure the selected PROMs align with the underlying framework and accurately capture the intended outcomes.

1.3.4 Quality of Life

1.3.4.1 EQ5D

A UK consensus for a core outcome set for clinical trials of patients with hip fracture, recommends the use of the EQ-5D as a minimum outcome measure.(75) To make informed healthcare decisions on the efficacy of treatments or interventions, or impact of disease or injury, an assessment of a patient's health needs to be measured. One method is to observe length of life and the other is quality of life. While length of life is easy to measure and is objective and binary, quality of life is more subjective. However, it can be measured by asking patients standard questions about different aspects of their health such as the EuroQol-5D (EQ-5D). It is a questionnaire that is used world-wide and is short, simple and is one of the worlds popular measures of health outcomes. It was developed by the EuroQol group an international network of researchers, made available by the EuroQol research foundation, a registered charity in the Netherlands. The EQ-5D measures five dimensions of health, mobility, self-care, usual activities, pain and discomfort and anxiety and depression. Patients tick boxes to show how they're feeling in each of these dimensions. They also rate their overall health using a visual analogue scale. This provides a description of a state of health. This description is then converted into numbers, which can be used to inform health care decisions. It is available in more than 200 languages and its flexibility allows it to be completed with paper and pen, digitally, through the phone, in face-to-face interviews or through care givers. The EQ5D is evolving, the original gave three response levels to choose from in each dimension. The new EQ-5D offers five levels to pick up more subtle differences in health. Since the EQ-5D is generic it is versatile enough to compare health across different types of patients, diseases, and treatments. Extensive research show it is a robust reliable responsive way of measuring health in a wide range of conditions and populations and the EQ-5D

simplicity makes it easy to use in routine measurements, health surveys and clinical trials.

A recent study compared the effect of a responsiveness and associations between a hip specific measure (oxford hip score), a measure of capability of older people (ICEpop CAPability) and a general health quality of life measure (EQ5D) from two large prospective studies. These outcome measures were studied at fixed timepoints in a consecutive sequence of patients. The ICECAP-O was not responsive to change, whereas the EQ-5D and the Oxford Hip Score were sensitive to change from baseline for both datasets. They demonstrated the EQ-5D could be used to measure outcomes for patients recovering from hip fracture, including those with cognitive impairment. It was also a moderately good predictor of death at twelve months. Following surgery and when reported by proxies such as relatives and carers it behaves similarly to self-reported scores and is also strongly correlated with hip specific scores.(83)

Haywood et al in 2016 undertook a systematic review of the quality and acceptability of PROMs in hip fracture. They found 28 PROMs were used in 71 studies, with SF-36 and EQ-5D 3L being the most widely used with acceptable evidence of measurement of properties. But there were limited evaluations of practical properties or relevance to this group of patients. For the remaining measures the evidence was limited. This study demonstrated the paucity of robust evaluation, and clear recommendations could not be made for PROM based assessment.(84)

1.3.4.2 Short form survey

The 36-item short form survey (SF 36), is a self-reported measure of quality of life.(85) It comprises 36 questions covering eight domains of health.(86) It is used in clinical practice and research and has been shown to be reliable in schizophrenia and childhood cancer and has been also validated in patients with stroke and mobility disability in the elderly. It was originally designed as a generic health measure but has been applied to specific diseases.(87) Individuals are required to complete the questionnaire. Scores are converted from the different domains and converted using a scoring key. Although components of the scores can be tallied combining physical

and mental components to form a summary, studies have shown interpretation can be difficult when performing this, (88) and literature also suggests it should not be used to confer an overall singular health related quality of life as it measures two dimensions mental and physical.(87)

1.3.5 Functional mobility

1.3.5.1 Hip Sprint Audit

Mobility is important to reduce complications such as pressure sores, DVT and PE, muscle deconditioning and chest infection. Patients who are active on the day of or after hip surgery are twice as likely to be home within a month.(89) The Hip Sprint Study in 2017, commissioned by the Chartered Society of Physiotherapists and managed by the Royal College of Physicians established 10% of patients were unable to get up out of bed day-1 post operatively. Their recommendation in the acute setting is to undertake early mobilisation with collaborative multidisciplinary working to reduce pain, postural hypotension, delirium and deconditioning of muscle so not prevent progress with physiotherapy.(90)

1.3.5.2 Rivermead Mobility Index

The Rivermead Mobility Index (RMI) assesses subjective functional mobility in gait, balance and transfers and was developed in 1991 to be used after head injury.(91) It is mainly used in patients with stroke, acquired brain injury, lower limb amputation, and multiple sclerosis. The RMI can be used for a wide range of functional ability levels, and those with cognitive impairment. It consists of 15 items 14 self-reported with 1 directly observed. Each item is scored 1 or 0 with a maximum total score of 15. A higher score indicates a better functional mobility. It has been shown to demonstrate excellent test-retest reliability in chronic stroke and lower limb amputees,(92, 93) and excellent for interrater and intra-rater reliability in acute stroke.(94)

1.3.5.3 Barthels Activity of Daily Living

The Barthels Activity of Daily Living (BADL) is an ordinal scale which objectively measures a person's ability to complete activities of daily living.(95) It was created to measure disability in patients whose rehabilitation impairments affect the use of their limbs to complete activities of daily living. Formal training is not required, the examiner just needs to be familiar with the functional skills being assessed and the scoring system used. Any member of the multidisciplinary team can perform this assessment, it takes only minutes to complete and can form part of a rehabilitation evaluation. It is highly recommended in individuals with stroke, Parkinsons' brain injury, cancer, COVID-19, patients admitted to intensive care and older persons. Despite multiple versions and adaptations to the index being available research supports the original Barthels Index as a measure and indicator of hospital length of stay, inpatient mortality, and discharge destination.(96)

Studies have shown the Barthels Index to have excellent internal consistency, excellent test-retest reliability and inter-rater reliability, and adequate inter-rater reliability.(97) All versions are however limited as they do not account for situational or environmental factors such as ramps, stair railings and bed adjustments. Research suggests conducting the assessments in environments best simulating the discharge environment to prevent falsely lowering or raising the score.(98) The BADL has been used extensively in research in patients with hip fracture and used as a measure of activity of daily living following comprehensive geriatric care multimodal treatment in patients with hip fracture.(99)

1.4 Frailty and hip fracture.

Frailty significantly impacts the risks, complications, recovery, and mortality associated with hip fracture. Frail individuals are more susceptible to hip fractures secondary to muscle weakness, impaired balance, and mobility.(100, 101) These individuals also have a higher complication rates following hip fracture compared to non-frail individuals. This may include infections, venous thromboembolism and pressure sores making them less able to cope with the stress of surgery and

recovery.(82). Due to initial low functional reserve in patients with hip fracture these patients may find it more difficult to regain their pre-injury level of independence.(102)

However, limited studies show the impact of frailty on recovery and outcomes following hip fracture, and quantifying frailty can be difficult using cumulative deficit models(102). With the quantification of frailty remaining a subject of substantial debate it requires further investigation to understand the biological basis of frailty to explain why those who are frail have poorer outcomes than those who are not frail following this devastating injury.

1.4.1 Diagnosis, prevalence, and pathogenesis of frailty.

Forecasts from the United Nations show the number of people above the age of 65 was 703 million in 2019, this is projected to increase to 1.5billion by 2050 so that 1 in 6 people in the world will be aged above 65.(103) Between a quarter and half of those aged above 85 are estimated to be frail with an increased risk of falls, disability, long term care and mortality.(104, 105) This causes profound implications for the planning and delivering of health and social care services. The most common cause of death in a 10-year prospective cohort study of community dwelling elderly people was frailty, followed by organ failure, cancer and dementia.(106)

With ageing there is a gradual decrease in physiological reserve, but in patients who are frail this is accelerated, with failure of homeostatic mechanisms it is important to therefore consider the complex mechanisms of ageing and how they promote cumulative decline in differing physiological systems. These complex systems can be influenced by genetic and environmental factors.(107) Epigenetic factors may also contribute through gene expression in cells, and could also be important in ageing. (108, 109)

Frailty develops because of age-related decline in physiological systems. It is a state of increased vulnerability to poor resolution of homeostasis after a stressor event. An

apparent small challenge such as a urine infection or injury results in a disproportionate change in health state, i.e., from independent to dependent, mobile to immobile, maintaining postural stability to falling, or lucid to delirious. These oscillations are seen the frail elderly populations have been referred to as unstable disability.(110) A fit elderly individual may decline in function but following this will recover to the previous functional state. A frail elderly individual after the same event may decline from independent to dependent, but their recovery may not return them to the same level of independence.

Frailty can frequently be identified through falls, delirium, and fluctuating disability. Fluctuating disability is a term used to describe day-to-day instability, such as 'good days' where a person may be independent and 'bad days' where a person may need extra care. Delirium is characterised by rapid onset of fluctuating confusion and impaired awareness. It reduces the integrity of brain function and is associated independently with adverse outcomes.(111) Balance and gait impairment are a major feature of frailty and a high-risk factor for falls. A 'hot fall' is related to a minor illness which reduces the postural balance below a crucial threshold necessary to maintain gait integrity. Spontaneous falls occur more in severe frailty when vital postural systems such as vision, balance and strength are no longer consistent with safe navigation through undemanding environments. Spontaneous falls are typically repeated and closely associated with the psychological fear of falling which leads a patient to develop severe impaired mobility.(110)

Ageing is the result of a lifelong molecular and cellular damaged caused by a myriad of mechanism regulated by a complex network of maintenance and repair. The threshold of damage to occur to cause impaired organ physiology is unknown, but many organs show redundancy which provides the physiological reserve to compensate for age and disease related change. The brain and skeletal muscle contain more neurons, and myocytes needed for survival.(112) This leads to the question, does a crucial threshold of age-related cumulative decline beyond which frailty becomes evident, exist in physiological systems?

A cross sectional study of 1002 women demonstrated an abnormality of three or more systems were a strong predictor of frailty.(113) This study demonstrated a non-linear relationship between the number of abnormal systems and frailty, independent of age and comorbidities. But the number of abnormal systems were more predictive of frailty than the abnormalities of any system. Therefore, when physiological decline reaches an aggregate critical level, frailty becomes evident. Frailty has been associated with the loss of physiological reserve in many systems including respiratory, cardiovascular, renal, haemopoietic and clotting systems. However, the brain, endocrine, immune system and skeletal muscle are the best studied in the development of frailty as they are intrinsically related.(113)

Frailty is both associated with increased delirium and subsequent reduced survival. A temporal association also exists between frailty and cognitive impairment and dementia. Elderly people without cognitive impairment at baseline frailty was associated with an increased risk of development of mild cognitive impairment for 12 years during follow up. Increasing frailty is associated with an increasing rate of cognitive decline.(114) Changes to Insulin-like growth factor (IGF) signalling via growth hormone, sex hormone and cortisol secretion are important in frailty although exact association are uncertain. The immune system in the elderly may function normally, but in the presence of acute inflammation may fail to respond adequately. Several inflammatory cytokines have been independently associated with frailty, such as interleukin 6 (IL-6), C-reactive protein (CRP), tumour necrosis factor alpha (TNFα) and CXC chemokine ligand 10 (CXCL10) .(115-118) Inflammation is associated with anorexia, catabolism of skeletal muscle and adipose tissue, which can contribute to nutritional compromise, muscle weakness and weight loss characterising frailty. Frailty has been associated with impaired antibody response to influenza and pneumococcal vaccine, which can explain the observation that vaccination is only modestly clinical effective in the elderly.(119)

A systematic review of 240 studies from 62 countries and territories, representing 1,755,497 participants reported prevalence proportions of frailty. Pooled prevalence in studies using physical frailty measures was 12%, compared with 24% for the deficit

accumulation model (those using a frailty index). For physical frailty, the prevalence was higher among females, 15%, than males, 11%. For studies using a frailty index, the prevalence was also higher in females 29% versus 20% for males. (120)

1.4.2 The models of frailty; cumulative deficit and phenotype models.

1.4.2.1 Phenotype model

The two main models of frailty include the phenotype model and the cumulative deficit model. Fried et al – conducted a secondary analysis of data obtained from a prospective cohort study (the Cardiovascular Health Study (CHS)) of 5210 men and women aged 65 years and older.(104) They established a frailty 'phenotype' with five variables; unintentional weight loss, self-reported exhaustion, low energy expenditure, slow gait speed, and weak grip strength. The lowest quintile values were used to define absence or presence of these variables. People with Parkinson's, stroke, cognitive impairment, or depression were excluded. Those with 3 or more of these factors were frail, those with 1-2 of the factors were pre-frail. Those with none of the factors were considered not be frail or robust. The population was categorised as 7% frail, 48% prefrail, and 46% not frail. Follow up was taken at 3 and 5 years, with the outcomes of falls, mobility and function, hospitalisation, and death. Those who were 'frail' developed a greater number of adverse outcomes than those who were pre-frail or not frail. Mortality at 7 years was 12%, 23% and 43% for not frail, pre-frail and frail respectively. The 7-year adjusted hazards ratio for mortality was 1.63 (95%CI 1.27-2.08) for the frail group.(104) However, although the work was important to suggest frailty phenotype can be defined and could potentially be a basis for detection of frailty in routine care, the way in which the variables relate to clinical practice is not clear. The 5 variables in this study were fortuitously available and selected from a prospective cohort not designed to investigate frailty. Cognitive impairment which is highly prevalent condition associated with functional decline and disability was not included as part of the phenotype. The general approach of clusters of variables to define frailty phenotype has however been independently validated.(121, 122)

1.4.2.2 Cumulative deficit model

The frailty index was developed as part of the Canadian Study for Health and Ageing (CSHA) study which was a 5-year prospective cohort study (n=10263) designed to investigate the epidemiology and burdens of dementia in the elderly in Canada. 92 baseline variables of symptoms, signs, abnormal laboratory values, disease states and disabilities were used to define frailty. The frailty index was a calculation of the presence or absence of each variable as a proportion of the total (20/92 deficits present = 0.22). Thus, frailty in this instance is defined as the 'cumulative' effect of individual deficits 'the more individuals have wrong with them the more likely they are to be frail'.(123)

The frailty index demonstrated a ɣ- statistical distribution. This is consistent with a probability model that typically describes systems with an in-built redundancy. This indicates that the frailty index has properties that fully support the idea of reduced homeostatic reserve. Thus, although every individual deficit carries no obvious or imminent threat of mortality, the deficits contribute cumulatively to an increased risk of death.(124) This is consistent with the idea that the increased vulnerability and threat of impending homeostatic failure is essential to the notion of frailty. This model experiences a theory of gradation, whereby frailty can be graded, which is more useful than a simple binary classification. Through grading frailty each grade has an equal weight in mathematical modelling. By grading frailty through an accumulation of deficits rather than a cluster pattern a relationship between adverse outcomes becomes apparent.

The cumulative deficit model expresses the theory of a grading of frailty each of which has an equal weight in its modelling. A value of 0.67 identifies an amount of frailty beyond which further deficit accumulation is not sustainable and death is likely.(125) Subsequent work has shown the 92 variables can be reduced to 30 without the loss of validity. The Frailty index is strongly linked to risk of death and institutionalisation, and the 10-year adjusted hazard ratio for mortality is 1.75 (95%CI, 1.41-1.74) for the frail group which is statistically similar to the phenotype model. Mortality is exponentially related to the value of the index.(125)

Both the phenotype and cumulative deficit model demonstrate overlap in identification of frailty. Demonstration of the convergent predictive validity for adverse health outcomes between two conceptually different models advances the debate whether frailty is a syndrome or a state. The continuous frailty index demonstrated greater discriminatory ability for people with moderate to severe frailty compared with the phenotype model, which has been validated independently.(126)

Although frailty is an abstract concept, levels of frailty can change and transition to a worsening level of frailty is more common than is the improvement to baseline following a physiological insult. The development of frailty leads to a spiral decline on increasing frailty and higher risk of adverse outcomes, such as worsening disability, falls, hospital admissions and death. Risk of admission to long-term care in those who are frail is greater than those who are not frail.(104, 127, 128)

1.4.3 Frailty indexes and scoring systems.

Frailty instruments can be classified as either self-reporting or performance-based instruments.(129) Instruments based on performance provide more precise and valid answers. (130, 131) Although they are less influenced by socio-demographic variables, personality, and cognitive factors. They are more sensitive to non-response, variations in time and difference in activity execution. They are also more difficult and time consuming to undertake.(130-132) Self-reported measures are believed to be an effective method for reaching to a large group of people achieving high response rates and providing reliable and valid responses.(132)

1.4.3.1 Comprehensive geriatric assessment

Comprehensive geriatric assessment (CGA) is a multiple step, multidimensional holistic assessment of an older person.(133) It evaluates their health and well-being and helps to formulate a plan to resolve issues which may concern the individual or their family and carers. Treatments are formulated which support the plan and at appropriate intervals the plan is reviewed and reassessed, through a multidisciplinary

approach involving physiotherapists, occupational therapists, nurses, families, and carers. Interventions are then adjusted accordingly.(133) The CGA involves a physical assessment, functional social and environmental assessment, psychological components, and a medication review. Older patients admitted to hospital who have undergone CGA have been shown to have a lower mortality and better independence (remaining at home) than those receiving standard care.(133, 134)

Within the community setting CGA along with complex interventions can reduce admission and readmission rates. In most cases the CGA is completed by the general practitioner at home or in the care home when the individual presents with; a fall, confusion, reduced mobility, incontinence or if the ambulance service has been called. It is most effective when completed by someone the patient and family trust and can help with advanced care planning.(133) Ideally it is completed proactively with several tools used for risk stratification to help monitor changes in frailty. Undertaking CGA however is a time-consuming process and to adequately complete it can take up to two hours and therefore assessments may need to be completed by several healthcare professionals with contributions from voluntary care sector and informal carers to accumulate the information required over time. A systematic review demonstrated there is uncertainty as to whether the data demonstrates a difference in effect between wards and teams during hospital admission and there is also imprecision and inconsistencies for the cost effectiveness in the hospital setting and it may result in a cost increase.(134)

In the UK the CGA forms part of the 'best practice tariff' and national benchmarking expects that amongst a range of other interventions a CGA will be undertaken for each admission as part of best clinical practice.

1.4.3.2 PRISMA-7

Since CGA is a resource intensive process directed by a geriatric medicine specialist several uncomplicated screening tools for frailty have been developed and endorsed by the British Geriatric Society.(135)

The Programme of Research to Integrate Services for the Maintenance of Autonomy (PRISMA -7) was created by a team in Canada as part of a large Canadian study as a case finding instrument to identify vulnerable individuals in the community.(136) It is a seven-item dichotomous questionnaire of which a score under 3 is indicative of frailty. Clegg et al analysed nine simple frailty screening instruments on community individuals and found PRISMA-7, slow gait speed and timed up and go test, showed a high sensitivity but moderate specificity to identify frailty. With a limited specificity implying false positive results they concluded these assessments should not be used as single tests alone to identify frailty. Based on these findings the British Geriatrics Society recommends all interactions between health and social care professionals and older people in the community includes a frailty assessment with PRISMA-7 being one of the tools which can be used. (133, 137)

Few studies have assessed the PRISMA-7 assessment in the hospital setting. It has been evaluated against other screening tools in the emergency department setting, and found to be the most accurate in distinguishing frail from non-frail patients.(138) It did not demonstrate a statistically significant difference when compared with the clinical frailty scale which can stratify patients to different levels of frailty based on clinical assessment. But due to its objective nature may be more appropriate than the CFS in this setting due to the severe time constraints within the emergency departments to undertake a CFS assessment. The CFS has been demonstrated to exhibit a ceiling effect when used in this way.(102) Another study however, has demonstrated no significant predictive value for PRISMA-7 as a predictor of stay in patients admitted to an acute geriatric ward.(139) No study has looked into the use of the PRISMA-7 and patients with hip fracture however, it has been used in the assessment of frailty for upper limb and vertebral fractures.(140, 141)

1.4.3.3 The frail elderly functional questionnaire

The Frail Elderly Functional Assessment (FEFA) is a questionnaire designed to assess function in frail elderly at very low activity levels. The assessment additionally focuses on areas that impact on caregiver burden. Questions address mobility, dealing with meals, dressing, and bathing, ability to answer the phone or take medications. The

questionnaire can be divided into higher functional tasks such as meal preparation or handling finances and psychomotor skills such as dressing and bathing. Inability to perform more basic functions lead to a higher score indicating a greater functional impairment. This questionnaire has been shown to be valid when administered over the telephone and through a proxy caregiver and is responsive to functional change. It is more discriminating between lower levels of function. Since it is not a performance-based measure it requires less time and space to perform and can be administered in most settings. Although it has the capacity to classify people as frail it does not distinguish between higher levels of function.(142)

1.4.3.4 Groningen frailty indicator

The Groningen Frailty Indicator (GFI) is a validated 15-item self-reporting questionnaire widely used in the primary care setting.(143) It assesses physical, cognitive, social, and psychosocial domains. Greater scores indicate higher frailty. A score of 4 is the cut off for frailty. It reflects current problems in patients' daily lives. It has high internal consistency and construct validity when compared with the Tilburg frailty indicator and Sherbrook postal questionnaire.(144) It has moderate overlap in the identification of frailty in the primary care setting when compared with the frailty index. However, as it is a self-reported questionnaire there may be variation in the self-management abilities of the patient and certain coping strategies or cognitive impairment might prompt the patient to report fewer problems than what may exist. Frailty as measured by GFI has been shown to be negatively associated with health status, self-rate health and capability in elderly patients 1-year following hip fracture.(145)

The GFI has been developed carefully and previous studies have shown it discriminates well between elderly subgroups especially in the oldest old, institutionalised patients, those who have a physical morbidity and those with a low income.(144, 146) Several studies have demonstrated a total GFI score in specific elderly populations which can be used to compare frailty scores between studies and by assessing frailty at multiple time points the dynamic status of frailty can be captured.(144, 146)

The GFI has been utilised in patients with hip fracture to observe the effects of quality of life up to 1 year following hospital admission. It has demonstrated frailty is negatively associated with health status following fracture.(145)

1.4.3.5 Tilburg frailty indicator

The Tilburg Frailty Instrument (TFI) is a tool for assessing frailty in older persons living in the community. It was created in the Netherlands by Gobbens et al based on a comprehensive literature review and the opinions of an international group of frailty experts, including geriatricians, gerontologists, nurses, and psychologists.(147)
It is a self-reporting instrument that older individuals must complete on their own.(148)

The TFI consists of two parts: part A, which focuses on 10 determinants of frailty, and part B, which focuses on 15 components of frailty.(148) The determinants are gender, age, marital status, education, income, ethnicity, lifestyle, life events, multiple diseases, and the living environment. Physical frailty (eight), psychological frailty (four), and social frailty (three) are the components of frailty. Physical frailty is characterised by poor physical health, unexplained weight loss, difficulty walking, difficulty sustaining balance, poor hearing, poor vision, a lack of hand strength, and physical fatigue. Memory issues, a depressed mood, an apprehensive or anxious disposition, and an inability to cope with problems are the components of psychological fragility. Social fragility is characterised by living alone, a dearth of social connections, and an absence of social support. The total TFI score ranges from 0 to 15, with scores between 0 and 8 for physical frailty, 0 and 4 for psychological frailty, and 0 and 4 for social frailty. Higher scores indicate higher frailty, as frailty is defined as a total TFI score above 4 in older adults.(148)

Two recent systematic reviews published, indicated the TFI is very suitable for assessing frailty among community-dwelling older people.(149, 150) According to Pialoux et al, the TFI and the SHARE Frailty Index(151) are potentially suitable for screening frailty in older people in primary care settings.(149) Sutton et al concluded

that the TFI has the most robust evidence of reliability and validity of 38 frailty assessment instruments, including frequently used instruments, such as the Phenotype of Frailty(104) and the Frailty Index.(150) However, both these systematic reviews conclude that further in-depth evaluation of the measurement properties of TFI is needed. Moreover, these previous reviews do not cover all available evidence due to their completion date and focus on evaluating several measurement instruments rather than on a single instrument.

A further systematic review that included a greater number of studies without language restrictions revealed that the TFI lacked evidence for a number of pertinent measurement properties.(152) It discovered moderately adequate evidence for TFI content validity, but insufficient evidence for the comprehensiveness of its items. The construct validity of the TFI was supported by sufficient evidence from two investigations of its structural validity and multiple hypothesis-testing studies with inconsistent results. No studies assessed cross-cultural validity or responsiveness of TFI scores. One of the three dimensions of the TFI demonstrated ample evidence for the internal consistency of its scores, while test-retest reliability results were inconsistent. The TFI demonstrated high concurrent validity with the comprehensive geriatric assessment. Several studies evaluated its predictive validity for adverse frailty-related outcomes, but the majority of their evidence was insufficient.(152) There is a need for evidence regarding the content validity of the TFI, particularly in regard to the comprehensiveness of its items from the perspective of frail individuals.

1.4.3.6 Edmonton Frail Scale

The Edmonton Frail Scale (EFS) is used in both research and clinical settings.(153) It consists of a checklist of nine domains which are cognition, general health status, functional independence, social support, medication use, mood, nutrition, urinary incontinence, and functional performance. It has been validated in both the inpatient and outpatient settings(154) and when compared with the frailty assessment of a geriatrician it demonstrates a strong independent association with high inter-rater reliability and internal validity. Construct validity was also comparable to the Barthel Index.(155) EFS total score 0-3 indicates no frailty, 4-5 points indicate pre-frailty, 6-8

points indicates frailty, 9-17 points indicate severe frailty. The EFS has not been completed for patients with hip fracture due to the fact the functional performance part of the assessment requires the patient to undertake a timed up and go test. A test which a patient with a hip fracture would be unable to perform on admission.

1.4.3.7 Clinical Frailty Scale Score

The Clinical Frailty Scale was introduced in the second clinical examination of the Canadian Study of Health and Aging (CSHA) to summarise the overall level of fitness and frailty of an older adult after they had been reviewed by an experienced clinician.(156) Initially used as an epidemiological tool it quickly evolved for clinical use to screen for frailty and stratify degrees of frailty and fitness. Initially a 7-point scale with time this evolved to 9 points due to both ceiling effect and the need to distinguish between severe frailty and terminal illness which were initially grouped together.(157) Descriptions of the scale have also been clarified with CFS 2 changing from well to fit, CFS 4 vulnerable to "living with very mild frailty" and levels 5-8 "Living with…" mild, moderate severe and very severe frailty respectively.(42) Patients with dementia does not limit the ability to score patients and it can be completed by non-geriatric clinicians (Table 1.3).

In recent years in the UK, the Acute Frailty Network(158) has promoted the widespread adoption of the use of the Clinical Frailty Scale (CFS) in all older patients admitted to hospital. The CFS is an assessment of a patient two weeks prior to their admission and is only validated in those above the age of 65.(156) A score is given once a comprehensive history and examination is completed, this is verified with a collateral history where appropriate and cross referenced with observations from other health care professionals. Functional assessments such as the ability to complete higher order tasks (e.g., maintain self-care and cooking) is also observed to allocate the CFS score accurately.

The British Orthopaedic Association Standards for Trauma (BOAST) The care of the older or frail orthopaedic trauma patient highlights the importance of undertaking a comprehensive geriatric assessment on patients who present with a clinical frailty

scale score of 5 or more. Therefore, it has become common practice for trauma and orthopaedic departments to undertake a clinical frailty scale score assessment of all patients admitted with a fragility hip fracture.(159) Later in this chapter we discuss the first large scale study to be undertaken using this frailty scoring system to predict outcomes specifically in patients with fragility hip fracture.

Table 1.3: The Rockwood Clinical Frailty Scale, adapted from Rockwood et al.(156) Abbreviations: ADLs, activities of daily living.

Category	Descriptor	Features
1	Very fit	Robust, active, energetic, motivated. Commonly exercises regularly. Amongst fittest for age.
2	Well	No active disease symptoms, but less fit than category 1, with less regular exercise or only seasonally very active.
3	Managing well	Medical problems well controlled, not regularly active beyond routine walking.
4	Vulnerable	Not dependent on daily help. Symptoms often limit activities. Feel 'slowed up' or tired during the day.
5	Mildly frail	More evident slowing, need help in high-order ADLs. Typically, impaired shopping, walking alone outside, meal preparation, and housework.
6	Moderately frail	Need help with all outside activities and keeping house. Difficulties with stairs, bathing and to a lesser extent, dressing.
7	Severely frail	Completely dependent on others for personal care (from either physical or cognitive cause)
8	Very severely frail	Completely dependent and approaching end of life
9	Terminally ill	<6 months life expectancy, but who are not otherwise evidently frail.

1.4.3.8 Electronic Frailty Index

The frailty index is routinely used throughout the world to quantify an individual's level of frailty. Within the National Health Service in England an electronic form is used. It emphasises the quantity of deficits, followed by their nature. One of the advantages of this method is that it can be constructed using existing clinical and population-based data. The frailty index is replicable across multiple databases because as a state variable it takes advantage of the high redundancy of the human organism. It can also be applied to almost any health dataset, is a comprehensive health assessment, and provides a continuous score ranging from fit to severely frail. Compared to other tools, it is more sensitive to alterations and has no ceiling or floor effect. However, the main challenge is it requires thirty variables to be included and if the data is not already included it can be onerous to collect.(160, 161)

The frailty index outperforms chronological age as a predictor of adverse outcomes, including mortality, disability, and cognitive decline. The distribution of frailty indices is unbalanced in community-dwelling samples (most individuals are healthy and have low frailty index scores). Individuals do not exceed a limit of 0.7 in the frailty index, this appears to reflect the extent to which individuals can tolerate health problems. The frailty index does not only increase with age; but can decrease, indicating enhanced health. Men have lower mean frailty index values than females of the same age, whereas females with the same frailty index value have a higher mean survival rate than males with the same index value.(160, 161)

1.5 Sarcopenia and hip fracture

1.5.1 Diagnosis, prevalence, and pathogenesis of sarcopenia.

Multiple studies have shown the presence of sarcopenia (the physical component of frailty), in patients who have sustained a fragility hip fracture have a worse prognosis than those who do not have sarcopenia. Patients have a poorer mortality at 1 year, have a lower functional capacity on admission and following surgery at 6-weeks, 3-months and 1-year, increased delirium post op, and increased institutionalisation. (162-165) However these studies varied on their assessment of sarcopenia based

upon different working group cut offs of sarcopenia and some even used their own cohort of recruited patients to define cut-off measurements for sarcopenia.

Sarcopenia is a progressive and generalised skeletal muscle disorder involving accelerated loss of muscle mass and function. Sarcopenia leads to falls, functional decline, frailty, and mortality.(166) It is an age-related process in the elderly influenced by coexisting risk factors over a person's life, including genetic and lifestyle factors. Initially described in 1980 as 'poverty of the flesh' the initial definition was based upon a decline in lean muscle mass resulting in reduced mobility, nutritional status, and independence.(167) Since then there have been two major developments in the definition. The first in 2010 where six consensus definitions added muscle function. Muscle function represented by muscle strength, power or performance was deemed to be more predictive of outcomes than muscle mass. The second major development was the introduction of sarcopenia to the International Classification of Diseases-10 code in 2016.(168-173) Sarcopenia can be better understood as skeletal muscle failure or insufficiency. It can appear acutely (through acute disease or sudden immobility) or as a chronic condition. Muscle mass along with bone mineral density peaks in young adulthood and after a plateau begins to decline with increasing age, with a faster decline in strength.(174, 175)

The World Health Organisation (WHO) has changed the focus of the provision of care in older people from a disease centred model to a function-centred model. Intrinsic capacity (a composite of physical and mental capacity of an individual) where interaction with the environment is used to determine functional ability. Muscle strength is included in the construct of intrinsic capacity which may warrant life-long monitoring.(176, 177) Sarcopenia can be associated with obesity, leading to disability and mortality. Sarcopenic obesity is defined by low muscle mass and increased adiposity are present in an individual. But in the care of obesity, sarcopenia may be unnoticed potentially leading to adverse outcomes. However, a consensus of the definition of sarcopenic obesity is yet to be agreed.(178)

Diagnosis of sarcopenia begins with an assessment of muscle strength via grip strength which has a well-developed protocol.(179) If grip strength falls below the reference value for the proposed society definitions, sarcopenia should then be suspected. However, the differential diagnosis is wide and other causes for reduced strength should also be considered. The next step in the diagnosis is an assessment of muscle mass.(180) Muscle mass can be measured in many ways, but all have limitations including variability in the results, inconsistent cut-off points and weak association between muscle mass and adverse outcomes. The most effective is the use of dual energy X-ray absorptiometry (DXA) which estimates lean mass. Other measures include bioelectrical impedance (BIA), computerised tomography (CT), magnetic resonance imaging (MRI) and ultrasound (US).

Muscle quality can refer to two different concepts, an association between strength and mass and observable characteristics such as intermuscular or intramuscular adiposity. Muscle quality may be more relevant than muscle mass, but it has not yet been sufficiently defined for use in clinical practice. Differing muscle mass cut-offs have a greater influence on diagnosis than muscle function cut off points.(181) The link between low muscle strength and adverse outcomes is well known. Two systematic reviews have identified an association between low grip strength and adverse outcomes in mortality, falls and fracture, cognitive decline, cardiovascular disease and admission to hospitals or institutionalisation. In the 1980s there has been an association with grip strength before hip fracture surgery and poor outcomes, and it has also been linked to frailty.(182) Newer consensus definitions of sarcopenia have been shown to have an association with falls, functional decline, frailty, and mortality.(183)

Most cases of sarcopenia are unnoticed, it cannot be universally screened for since the screening tools are not accurate and the effect of screening on outcomes are far from proven. Therefore, a case finding approach is adopted. This is particularly relevant in care setting where sarcopenia may be more prevalent such as hospital admissions, rehabilitation, and nursing homes. The SARC-F is a commonly used sarcopenic finding instrument it has a low specificity but high sensitivity. The screening

questions address strength, assistance in walking, rising from a chair, climbing stairs and falls.(184) Blood biomarkers are not yet available for diagnosing sarcopenia, research in this area is complex due to the differing views of the definition of sarcopenia, increasing recognition of acute and chronic sarcopenia and the existence of many interacting pathways.(185)

Once sarcopenia has been confirmed a systemic approach is recommended to ascertain the underlying cause. It can occur with a range of conditions and in most older patients it may be associated with more than one condition. When no cause of gradual onset sarcopenia is present in an elderly person, age-associated (primary) sarcopenia is diagnosed. The underlying causes of sarcopenia include nutritional (low protein or energy intake, micronutrient deficiency, malabsorption) inactivity (bed rest, deconditioning, sedentary lifestyle), disease (bone and joint diseases, cardiorespiratory disorders, metabolic disorders), and iatrogenic (hospital admission and drug related). It is therefore important to distinguish sarcopenia from, malnutrition, cachexia, and frailty.(186-188)

With sarcopenia there are strong recommendations for physical exercise programmes to help improve strength and reduce complications but with nutritional intervention the evidence is less consistent.(184) There are pharmacological interventions such as Vitamin D which has been shown to improve the strength and function in women. Testosterone supplementation has also been considered but the evidence is limited.(189)

1.5.2 Skeletal muscle and measurements of mass, strength, and physical performance.

A range of radiological imaging modalities can be used to evaluate muscle mass and quality to help diagnose sarcopenia. These include imaging modalities computerised tomography (CT), magnetic resonance imaging (MRI), dual-energy X-ray absorptiometry (DXA), and ultrasound (US). There are also a range of functional measures (e.g. timed up and go test, hand grip strength) that have also been

implicated as measures of sarcopenia. The chief modalities in research and clinical use are discussed.

1.5.2.1 Computerised tomography

CT can be used to assess muscle mass and quality.(190) It measures muscle size and attenuation in specific areas, which contrasts with DXA where a whole-body estimation of lean muscle mass occurs. Measurements from a single cross-sectional slice have been high correlated to whole body adipose tissue and skeletal muscle distribution.(191, 192) More commonly abdominal CT scans are used in studies on sarcopenia; scans are usually measured at the level of L3 or L4 where regions of interest are drawn around either the psoas muscle or all muscles on a single slice. Other studies have used the levels of T12 or C3, and in some instances CT scans have been undertaken of mid-thigh.(193-195) The choice depends on the series of patients and concurrent conditions they may have. The cross-sectional area of muscle is taken with height squared to provide the skeletal muscle index (SMI). Recent meta-analysis demonstrates the most common cut-offs for men is 52-55cm/m^2 and 39-41cm/m^2 for women.(193) CT parameters are directly influenced by degree of muscle fat infiltration. Currently there are no consensus standardised CT thresholds to be used to establish a diagnosis of sarcopenia.

CT scans demonstrate a high accuracy and reproducibility. They are routinely performed for patients with cancer as part of their staging and follow up. In research it therefore provides an opportunistic method to assess for sarcopenia retrospectively without the need for additional scans. CT scans have a high set up cost for research units, this can be negated if hospitals are linked to a research institute. In this case although access to a scanner is available reducing the set-up cost, often clinical need to undertake scans will take a priority unless slots are ringfenced for research. The units are not portable and therefore research subjects must be stable enough medically to be taken to the scanner. Once a scan is commenced it can be difficult to resuscitate a participant should the need arise. There may be contraindications to performing CT in some subjects including allergy to contrast, and conditions where participants are unable to lie flat. If the CT scans are measured opportunistically from

contrast CT this may provide measurements less accurate than non-contrast CT scans, since contrast affect tissue enhancement. Implants within the subject may also distort the images and measurements. If CT scans are used in patients who have cancer, it can be difficult to evaluate if the muscle loss or changes in muscle mass are because of sarcopenia or as a pathological result of the cachexia through malignancy. The scan requires the use of specialised staff to process the images using software which can be complex and there are no clear low muscle mass thresholds. Lastly there is a significant amount of radiation involved to undertake the scan and therefore is only currently used for research purposes.

1.5.2.2 Magnetic resonance imaging

MRI measures muscle and fat due to its high contrast resolution and multiparametricity. It demonstrates high accuracy through measurements of images of cross-sectional surface area and volume of muscle. It also shows a strong correlation with CT in body composition analysis on cadavers.(191, 196) However, studies using MRI have been performed on different muscles with no standardisation of image protocols or biomarkers.(197, 198) MRI can assess muscle quality, specifically muscle oedema, fibrous and fatty infiltration, and elasticity. It accomplishes this through multi-echo gradient sequences like the Dixon technique to quantify intramuscular fat.(199) Adipose and fibrous tissue are non-contractile muscle components increased in sarcopenia and can be detected on MRI through rate tensor imaging by assessing contractility and elasticity of muscle. Changes in rate tensor imaging show age related and regional differences.(200)

Diffusion tensor images originally used to track nerve fibres and cartilage imaging, allows assessment of microstructural changes in muscle fibre architecture altering water diffusion and fractional anisotropy.(200) These values are related to the directional orientation of water molecules in tissue which is affected by muscle fatty infiltration in sarcopenia.(200, 201) A recent study reported significantly higher fractional anisotropy and T2 values in the quadriceps muscles of pre-frail/frail patients (Fractional anisotropy = 0.32, T2 = 50.66 ms) compared with controls (Fractional anisotropy = 0.28, T2 = 39.48 ms).(201)

MRI scans if performed for patients for a clinical need provides an opportunistic method for assessing sarcopenia retrospectively without the need for additional scans. It demonstrates a high accuracy and reproducibility and is highly correlated with CT. A significant advantage over CT is the zero-radiation exposure and risk to participants and high spatial resolution. Analysis of muscle oedema and fatty infiltration can also be accurately assessed. Similarly, to CT the setup cost is high for research institutes. With a greater limit on availability compared with CT due to cost and resources. MRI scans require the use of specialised staff to process the images using software which can be complex and again there are no clear low muscle mass thresholds. MRI scanners are not portable and require the participant to remain still for extended periods of time when compared to CT. This is to obtain high quality images required for analysis. MRI may also be contraindicated in those with metal implants and pacemakers due to the magnetic fields of the scanner.

CT and MRI are currently the gold standard imaging modalities to assess muscle mass and quality, but no clear cut off values have been reported to identify sarcopenia, limiting the application of these modalities to research purposes.

1.5.2.3 DXA

DXA is a whole-body scan commonly used to assess body composition, lean mass, fat mass, and bone mineral content. When assessing fat mass and lean mass this has been shown to have a high correlation with adipose and lean mass tissue measure with CT and MRI.(190, 202, 203) It should be noted that lean mass is an estimation of all non-fat non-bone tissues. works by emitting X-rays at two different energy levels.

The sum of the upper and lower limbs lean mass is called the appendicular lean mass (ALM). This is generally used to measure muscle mass in DXA, this value is then indexed with height squared to give the appendicular lean mass index (ALMI = appendicular lean mass / height^2). The reference ranges are varied amongst consensus groups. DXA should always be integrated with clinical and physical performance data

and used to confirm a diagnosis of low muscle mass in patients with a clinical suspicion of sarcopenia.(166)

DXA has the advantages of being able to provide data on body composition, and a better evaluation of complex syndromes including osteoporosis, obesity and cachexia (204) and therefore the European working group on sarcopenia for older people (EWGSOP) suggest DXA as the first line tool in clinical practice above CT and MRI which, are to be used for research purposes. However, it has limitations, it requires education in correct exam acquisition, participant positioning, and image analysis to avoid errors in outcome.(192, 205, 206) It does not provide information on muscle quality such as fat infiltration, and measurements may be affected by hydration status. Again, it is not portable and requires transfer of participants to the scanner.

1.5.2.4 Ultrasound

Muscle mass (estimated measures using cross sectional areas or volume) and quality can be reliably assessed using US.(207) Muscle mass can be assessed using cross sectional surface area (CSA) and or muscle thickness, or muscle volume. Studies have demonstrated higher inter and intra-reader reproducibility of US parameters with good correlation with reference techniques.(208-210) High inter-reader reliability has been shown when comparing US with MRI in evaluating CSA of muscle, with no significant differences in measuring muscle CSA and volume.(208) However many studies use US on healthy controls and therefore report a low prevalence of sarcopenia.(209) The majority of studies perform measurements on the quadriceps muscles but, some have used postural muscles of the lower limb.(211)

US can provide additional information regarding muscle quality. The degree of intramuscular fat and connective tissue can affect the echogenicity which can be detected with US. Some measurements can be related to muscle function. Strength in elderly muscle has been correlated with vastus medialis thickness, and echotexture of the mean pixels intensity of rectus femoris.(212) Pennation angle and muscle fascicle length at rest or during contraction have been associated with the loss in maximum force and shortening velocity of muscle fibres seen in sarcopenia.(213, 214) Other

advantages include the use of portable scanners for measurements to be conducted at the bedside, no radiation risk to participants, no clinical contraindications such as metalwork or implants which may affect image quality, low cost, and high availability.(215) Data is however scarce when assessing for muscle stiffness and microvasculature associated with sarcopenia.(216, 217) Despite being widely used in many musculoskeletal conditions and having the aforementioned advantages it is operator dependent and at present there are no cut off values to evaluate muscle mass and quality(218) limiting its application in diagnosing sarcopenia.

1.5.2.5 Bioimpedance analysis

BIA is an affordable non-invasive test to identify for sarcopenia. The results are readily available and do not rely on skilled personnel. It estimates body composition by calculating the electrical conductivity amongst different tissues. Impedance (tissue resistance and reactance) is registered when low voltage current is passed through the body. The differences amongst devices which use BIA are prediction equations, number of electrodes, and frequency of alternating current. Accuracy depends on ethnicity, tissue hydration, food intake, and comorbidities. (219) Within the same patient these factors can influence the accuracy throughout different time points in the day. It is contraindicated in those with implanted devices. Multifrequency BIA (a combination of low and high frequency to calculate intracellular, extracellular and total body water has a greater accuracy than a single frequency BIA in obese and underweight individuals but these variables need to be accommodated when undertaking pre-test preparation to select the appropriate equation used by the software and determining cut off points for specific populations. Therefore, restrictions are put in place on participants regarding hydration, food consumption and exercise prior to BIA. (219) Further research is required in this area to see if it is viable in detecting low lean muscle mass and consensus is required amongst working groups regarding the cut-off points.

1.5.2.6 D3-Creatine dilution

A promising way of calculating total body muscle mass is the use of D3 creatine dilution method which has been validated in human studies. Creatine is present in 95% of skeletal muscle, 2% of creatine is converted to creatinine per day via irreversible nonenzymic mechanisms. Based upon this conversion being constant among and within subjects. The daily excretion rate of creatinine has been used as a metric of whole-body creatine pool size.

It directly measures total body creatine (Cr) pool size. This method measures the dilution of an oral dose of D3-creatine (D3-Cr) by assessing the enrichment of D3 creatinine (D3-Crn), representing the ration of administered labelled D3-Cr to unlabelled endogenous Cr in the body, after non-enzymatic conversion of Cr to creatinine (Crn). It assumes the tracer D3-Cr dose is absorbed and quantitatively transported to the skeletal muscle cells. Intramyocellular Cr is turned over through non-enzymatic conversion to Crn at the rate of about 1.7%/d and excreted in urine, thereby providing non-invasive access to the intramyocellular pool of labelled (exogenous) and unlabelled (endogenous) Cr.(220)

The human validation study demonstrated in some subjects a small amount of D3Cr dose was directly excreted in the urine following oral administration in most of the older women (1-5% loss) in the study when compared with the younger males.
This method requires the accurate collection of urine over a 24-hour period and repeat urine samples at specific times which can be difficult to achieve. Inaccuracies can be seen is If samples are missed or timings are not exact, or there are changes in temperature or pH which may affect the conversion rate of creatine to creatinine. (221) Low D3 muscle mass is strongly related to physical performance, mobility and incident injurious falls in older men.(222)

1.5.2.7 Hang grip strength.

Hand grip strength has been well documented to diagnose sarcopenia in elderly patients. It is a simple and low-cost test and can be performed at the patient's bedside. Consensus groups for diagnosing sarcopenia have informed to use hang grip strength

as the first test to diagnose probably sarcopenia.(223) The cut off values vary between working group definitions. The disadvantages include patients with hand pathology and cognitive impairment may find it difficult to undertake the test due to restriction of hand function or inability to follow instructions.(224)

Physical performance is defined as the ability to carry out physical tasks to function independently in daily life. It can be characterised using subjective or objective assessment of mobility, strength, and balance. It relies on the coordination of the central and peripheral nervous system with the musculoskeletal system and therefore it is a function of the whole body. Performance can be measured by gait speed or 400m timed walk. Composite measures such as the Short Physical Performance Battery (SPPB) and the Timed Up and Go (TUG) test can also be used.

1.5.2.8 Chair sit stand

The chair sit stand test requires a participant to sit in the middle of a chair without armrests and stand as many times as they can for 30seconds. It can be performed by any health care professional but requires the participant to be able to stand from a seated position on their own. It would be difficult for those who were admitted in an acute setting to a hospital to perform this test. For those who can perform the test it provides useful information on the strength of the proximal thigh muscles and has a good correlation with diagnosis of sarcopenia. Working groups have highlighted its use as a tool to diagnose probably sarcopenia prior to muscle mass and performance assessments. However recent literature has stated grip strength to be more of a powerful tool to predict sarcopenia than chair stand test.(223)

1.5.2.9 TUG/4m walk test.

The timed up and go (TUG) test is used as a performance measure in sarcopenia and falls risk. It requires the participant to stand from a seated position in a standard arm chair, walk three metres / 10 feet, at their normal pace, turn around walk back to the chair and then sit back down. It is a simple test with low cost.

The short physical performance battery (SPPB) is an objective measurement of balance, lower extremity strength and functional capacity in adults>65 It involves three phases walking sit to stand and balance.

Chair stand test requires the participant to stand from a seated position without using their arms. If they are successful, then they perform the repeated chair stand test which requires them to stand from a seated position 5 times as quickly as possible and the time is recorded. If the participant uses their arms to perform the chair stand test a score of 0 is given or if the test is not completed. Next a balance test is performed, the participant is asked to stand unsupported for 10 seconds with their feet in certain positions (feet together, semi tandem, and full tandem).

Gait speed test, this measure gait speed walks to a line 3/4 meters at normal pace
Studies have demonstrated excellent interrater reliability of the SPBB(225) and test-retest reliability in people with dementia also to be excellent.(226) In patients with multiple sclerosis it has shown to be associated with measures of lower extremity function and weakly associated with measures of upper extremity function.(227)

1.5.3 Sarcopenia working groups and the difficulties in diagnosis.

With so many investigations available, there are a range of different definitions of sarcopenia (and other body compositions methods). Body composition also varies by gender, race, and age and as such definition can be subject to opinion and debate. There are several widely used consensus definitions. This study utilised the European definitions, but the two major working groups are discussed below.

1.5.3.1 Asian Working Group for Sarcopenia

Diagnosis of sarcopenia in Asian populations requires consideration of the anthropometric and lifestyle factors when compared with western countries. For example, relative smaller body size, higher adiposity and less mechanised more physically active lifestyles. In 2014 the Asian Working Group for Sarcopenia (AWGS) proposed a diagnostic algorithm based upon Asian data. this assisted further research

into sarcopenia in the Asian continents and following this the algorithm were modified in 2019.(228) AWGS defined sarcopenia is significantly associated with increased risks of physical limitations at 4 years, slowness at 7 and 10-year mortality but not of hospitalisation.(229-231) The majority of studies in Asian countries have used the AWGS 2014 criteria of sarcopenia but following extensive deliberations the AWGS 2019 criteria plays an emphasis on measurements of muscle quantity and quality. The AWGS defines a person with low muscle mass, low muscle quality and low physical performance as having severe sarcopenia. Due to the difficulties in measuring muscle mass in the community the AWGS also support appendicular skeletal muscle mass using BIA.(228) The AWGS promotes the stratification of sarcopenia diagnosis to facilitate treatment and interventions and introduces 'possible sarcopenia' defined as low muscle strength with or without reduced physical performance.(228)

The case finding special interest group reviewed studies from east and southeast Asia which used screening or assessment instruments to identify the early stages of sarcopenia. Based upon validate reports the AWGS recommends using calf circumference or SARC-F questionnaire. AWGS recommends CC<34cm men and <33cm for women for screening or case finding.(228) The SARC-F questionnaire assess 5 components: strength, assistance in walking, rising from a chair, climbing stairs and falls. A score of ≥4 was defined as sarcopenia, SARC-CalF improved the sensitivity of the SARC-F by including calf circumference with a score ≥11 diagnosing sarcopenia.

Although CT, MRI, DXA and BIA have all been used to assess muscle mass the predominant modalities in Asia have been DXA and BIA. BIA with a multifrequency device correlated better with DXA-measured ASM than with other BIA devices. AWGS however does not recommend the use of BIA devices designed for home use due to suboptimal diagnostic accuracy. Although some studies have shown ultrasound measurements to be promising to estimate muscle mass the AWGS does not recommend US until more data is available. The AWGS therefore recommends the use of DXA or multifrequency BIA, both height adjusted, for measuring muscle mass in sarcopenia. The cut-off values are as follows: <7.0kg/m2 men, <5.4kg/m2 women by DXA; and <7.0kg/m2 in men and <5.7kg/m2 in women by BIA.(228)

Almost all studies in east and southeast Asia which focused on sarcopenia, muscle function and strength used hand grip strength to indicate skeletal muscle strength. Therefore, the AWGS 2019 retained the use of hand grip strength as a measure of muscle strength. The two main types of device used to measure hand grip strength are the spring type dynamometer (Smedley) and the hydraulic type (Jamar).(232) AWGS 2019 recommends the use of either, provided that the standard protocols for each are respectively followed. The data generated from these dynamometers are not directly comparable. Elderly individuals demonstrate a higher grip strength when using a Jamar device opposed to a Smedley. Therefore, the AWGS does not propose dynamometer-specific cut off points due to insufficient comparative data. The standard positioning recommended by the AWGs 2019 are standing with full elbow extension for the Smedley dynamometer and sitting with the elbow flexed at 90 degrees for the Jamar dynamometer.(228)Sitting is preferable if the participants cannot stand. The maximum reading of at least 2 trials is to be taken. Using either both hands or the dominant hand in maximum effort isometric contraction. Rather than fixed acquisition time. The AWGS recommends low muscle strength diagnostic cut offs of handgrip <28.0kg for men and <18.0 kg for women.(233)

AWGS 2019 recommends defining low physical performance based upon either SPPB, 6m walk or 5 timed chair stand test cut-offs; timed up and go is not recommended as the results may reflect complex pathoetiologies.(228) The AWGS 2019 recommends measuring the time taken to walk 6m at a normal pace from a moving start, without deceleration and taking the average result of at least two trials as the recorded speed. Most studies in Asia used AWGS 2014 cut-offs of ≤0.8m/s for the diagnosis of sarcopenia, but others have used criteria based upon their own cohort or previous publications. (234-236) Slow gait speed <1m/s predicts rapid cognitive decline in older men and higher dementia risk amongst community older adults.(237) Therefore AWGS 2019 recommends using gait speed to define reduced physical performance and has increased its cut-off from ≤0.8m/s to <1.0m/s. although some studies have reported sex differences in gait speed the AWGS 2019 does not recommend sex specific cut offs.

Impaired mobility defined as SPPB score ≤9 was more predictive of all-cause mortality in a systematic review.(238) The mean SPPB scores of older adults with AWGS-defined sarcopenia in a recent report was 10.5. Therefore, the AWGS recommends SPPB score ≤9 as the cut-off for low physical performance. The 5-time chair stand time had been proposed as a surrogate for gait speed.(239) A stand time of 11.6 seconds corresponded to a walking speed of 1.0m/s, hence AWGS recommends ≤12 seconds as the cut off for low physical performance.

1.5.3.2 European working group for sarcopenia for older people

The European Working Group on Sarcopenia in Older People (EWGSOP) published their original sarcopenia definition in 2010 which was widely used. In 2019 a revised definition was formulated (EWGSOP2) this was based on the accumulation of scientific knowledge over this period.(166) The original 2010 EWGSOP definition resulted in one of the lowest pooled prevalence estimates, with higher estimates coming from older definitions.(168) As the definition of sarcopenia evolves the estimations of prevalence is becoming more accurate.(240) Sarcopenia is more prevalent in hospitals or acute care settings, and care homes than in the community.(166) The latest from the EWGSOP2 definition of sarcopenia states the physical performance should be used to assess the severity of sarcopenia. Grading the severity is important for predicting outcomes and choosing the intensity of interventions. An intensive multidimension intervention that always includes exercise are needed for severe sarcopenia.

However, full agreement of the variables and cut off points are yet to be reached. The most widely cited definition proposed is from the European Working Group on Sarcopenia in Older People (EWGSOP), (since 2019 the EWGSOP has recently been updated as EWGSOP2).(166) supported by the Asian Working Group on Sarcopenia (AWGS).(228)

Only the EWGSOP2 definition is endorsed by a range of international scientific societies (European Geriatric Medicine Society EuGMS; The European Society for Clinical Nutrition and Metabolism ESPEN; The European Society for Clinical and

Economic Aspects of Osteoporosis, Osteoarthritis and Musculoskeletal Diseases ESCEO; International Osteoporosis Foundation IOF; and International Association of Gerontology and Geriatrics European Region IAGG-ER) for clinical practice and research.

The EWGSOP2 recommends the use of the SARC-F questionnaire, it is an inexpensive and convenient screening tool with a low to moderate sensitivity and very high specificity to predict low muscle strength(241) as such it will mostly detect severe cases. Since the SARC-F is self-reported by the patient its results may reflect perceptions of adverse outcomes that matter to the patient.

EWGSOP advises using grip strength and chair stand test to identify low muscle strength. To evaluate muscle quality and quantity in clinical care DXA and BIA is advised, and in research or specialties care for individuals at high risk DXA, MRI, or CT. For measures of physical performance SPPB, TUG and 400m walk is recommended to assess the severity of sarcopenia.

Typically, appendicular lean mass (skeletal muscle in the extremities) is estimated and adjusted for height to define cut off values. Research focussing on identifying cut-off values has led to a range of different cut off values that are difficult to reconcile and introduce. EWGSOP has taken a pragmatic approach in the updated definition and opted for simple cut off points which are easy to remember, exploiting the best available data. This may increase the potential uptake of the consensus definition to form a standardised approach to identify sarcopenia in patients.(166)

International working group for sarcopenia

International clinical practice guidelines for sarcopenia have come to the recommendation for the use of an internationally accepted measurement tool for sarcopenia including the EWGSOP and Foundation for National Institute of Health (FNIH) definitions and for rapid screening. A recent study has shown EWGSOP2 to have a higher predictive value on mortality at three years in older hospitalised adults.(242)

1.6 Overall aims and objectives.

The frail patient who sustains a hip fracture poses significant challenges to healthcare professionals and systems. The impact of the physical component of frailty on outcomes in this cohort of patient has been shown to be poor however the biological basis for these complications and poor mobility has not yet been investigated. Our aim was to investigate the biological basis for the physical component of frailty and the potential link to and causes for poor outcomes.

The aim of this study was to characterise the gene expression within vastus lateralis muscle of frail older people.

To determine whether.

- Measures of muscle mass +/- muscle strength are predictive of clinical outcomes in patients with hip fracture.
- There are associations between these estimates of muscle mass and
    - Grip strength
    - Indices of patient frailty (questionnaire derived)
    - Indices of patient mobility (questionnaire derived)
    - Mortality
- There is a difference in muscle gene expression between the damaged vs non damaged limb to provide an insight into the molecular mechanisms underpinning acute muscle decline associated with hip fracture.
- To determine if there is a difference in gene expression between non-frail and frail patients.
- To determine whether inflammatory status at the time of surgery impacts survival.

These aims and objectives form part of a wider study which is being undertaken called the Functioning of Elder Muscle Understanding Recovery (FEMUR) study.

2 The Rockwood clinical frailty scale score and hip fracture outcomes (30-day survival, post-operative complications, institutionalisation, and length of stay). Preliminary work

2.1 Overview

Preliminary work was undertaken which formulated the basis of this PhD. The clinical frailty scale has been adopted in widespread use in the assessment of frailty in patients with fragility hip fracture. Since its adoption it has also been important in the monitoring of the delivery of hip fracture care through the best practice tariff. We evaluated the use of this frailty system and its relationship with the frailty outcomes of 30-day morality, new institutionalisation, post operative complications and acute length of stay. There were linear and non-linear correlations which are discussed further highlighting the need for further investigation and research in the frail cohort to understand the biological basis of frailty and poor outcomes in these patients.

2.2 The Rockwood clinical frailty scale in clinical practice.

A significant number of patients in the UK present annually with hip fractures. Our institution, a 1700-bed, level 1 major trauma centre, admits roughly 900 hip fracture patients per year. The NHFS developed at our institution already provides patients with their 30-day mortality risk; however, it does not provide information on frailty outcomes such as post-operative complications, institutionalisation, and duration of acute hospital stay. When developing care plans, it would be beneficial for clinicians, patients, and their families to be able to distinguish between those at high and low risk of a poor outcome. Frailty status may provide the chance to do so. Two studies have shown that frailty indices are predictive of short(100) and long-term mortality(101) and hospital length of stay(100) after hip fracture, but neither study examined whether frailty measures predict other clinically significant outcomes associated with hip fracture, such as complications or institutionalisation. Since the widespread adoption of the CFS in acute hospital settings, we sought to determine

whether this frailty scoring system could predict frailty outcomes and identify areas where patient care could be further improved or investigated.

2.3 Methodology

In preparation for the main body of work undertaken as part of this thesis we undertook a prospective observational cohort study between April 2018 and December 2019 in our institution. This is a 1700-bed hospital group which includes the only regional major trauma centre. This work aimed to assess the value of the CFS as a predictor of outcomes and compare it to another commonly used frailty scale. This has since been published and is one of the only studies validating the CFS in the clinical setting.

All patients referred by the emergency department with a fragility hip fracture following either a low energy trauma, a fall, or inability to mobilise were included. Patients without a recorded CFS on admission, or those under the age of 65 were excluded. Data was collected during admission and inpatient stay by routine independent audit personnel using the Standardised Audit of Hip Fracture in Europe (SAHFE) collection form.(243) All data remained anonymous and was entered onto a local hip fracture database according to national data protection standards. Research ethics approval and informed consent was not required as this study was conducted under existing clinical governance procedures using the data for quality improvement. The index test was the CFS which was assigned by the admitting team of doctors (Core surgical trainees / Specialty registrars) trained in the management of hip fracture and frailty. The reference standard was the NHFS which is a validated measure and routinely used in our institution and nationally to predict 30-day mortality.

Our institution strictly adhered to a 'fractured neck of femur care pathway' that standardised care upon admission and throughout the inpatient stay. Patients were treated in accordance with national best practise guidelines. They underwent light anaesthesia, early surgery, and an orthogeriatric care and rehabilitation package. The following data was collected: demographic characteristics (age, sex, height, and

weight), Abbreviated Mental Test scores (AMTS), mobility status (freely mobile, mobilises with one aid, mobilises with two aids or a frame, some indoor mobility but no outdoor mobility, and no functional mobility), NHFS, admission blood results (including full blood count, urea, and electrolytes), and type of hip fracture (intracapsular displaced and Atrial fibrillation; asthma; anaemia on admission; blindness; chronic obstructive pulmonary disease; chronic kidney disease; cerebrovascular accident/transient ischaemic attack; dementia/cognitive impairment; diabetes; dysphagia; depression; alcohol excess; epilepsy; gastroesophageal reflux disease; heart failure; hypertension; ischaemic heart disease; liver disease; malignancy; obesity; paediatric aortic aneurysm; paediatric. The collected patient procedural data included mechanism of injury, date and time of admission, date, time, and type of surgery, duration of hospital stay, and, if applicable, date of death. In-hospital complications such as pulmonary embolus, pneumonia, urinary tract infection, cerebrovascular accident, myocardial infarction/acute coronary syndrome, deep vein thrombosis, dislocation, failure of fixation, blood transfusion, acute kidney injury, pressure ulcer, Clostridium difficile infection, and reoperation within 120 days were collected. Outcome data regarding 30-day mortality, inpatient postoperative complications, length of stay (LOS), and discharge (admission to residential care/nursing care) were also collected.(102)

Missing NHFS scores were calculated retrospectively, we felt this was deemed to be acceptable as the parameters to calculate the scores were collected prospectively. The CFS was not calculated retrospectively as it is a measure which may be subject to recall bias and therefore if CFS was not recorded these patients were excluded from the study.

Statistical analysis of the data was performed with SPSS Statistics for Mac v. 26 (IBM, USA). At the outset of analysis, we assumed no a priori relationship between CFS and our choice of outcomes. The first means of examining the relationship between the CFS and outcomes of interest was visual inspection of the data through bar graphs. Based on this visual inspection, the appropriate statistical tests were chosen. Tests included chi-squared test for categorical variables, analysis of variance for continuous

variables (ANOVA), and two-tailed Spearman's correlation test between own home to institution, and CFS. Receiver operating characteristic (ROC) curve and Kaplan-Meier survival analyses were performed.(102)

2.4 Results

From 1577 patients admitted with a hip fracture 1255 were analysed following application of the exclusion criteria. Baseline variables are demonstrated in (Table 2.1). With increasing CFS baseline parameters such as age, cognitive impairment, abnormal BMI, residential status, pre-injury mobility, disability, and comorbidities increased. Very few patients scored CFS 8-9

The relationship between CFS and each outcome differed. Mortality at 30-days increased linearly from CFS 1 to 5 but was stable from CFS 5 to 8 (Figure 2.1). Statistical testing confirmed a significant positive linear relationship between CFS 1 to 5 and 30-day mortality (Spearman's Rho 0.12; $p < 0.001$) and no significant relationship between CFS 5 to 9 and 30-day mortality ($rs(646) = 0.00$; $p = 0.980$) (Table 2.2). Visual inspection suggested that the incidence of postoperative complications similarly rose linearly from CFS 1 to 4 but was stable from CFS 4 to 8 (Figure 2.1). Statistical testing confirmed a significant positive linear relationship between CFS 1 to 4 and presence of postoperative complications ($rs(605) = 0.21$; $p < 0.001$), and no significant relationship between CFS 4 to 9 and postoperative complications ($rs(882) = -0.03$; $p = 0.376$). Visual inspection suggested that LOS rose linearly from CFS 1 to 4 but was stable from CFS 4 to 8 (Figure 2.1). Statistical testing confirmed a significant positive linear relationship between CFS 1 to 4 and LOS ($rs(589) = 0.31$; $p < 0.001$), and no significant relationship between CFS 4 to 9 and LOS ($rs(857) = -0.18$; $p = 0.594$). Visual inspection suggested that the incidence of institutionalization rose linearly from CFS 1 to 7 (Figure 2.1). Statistical testing confirmed a significant positive linear relationship between CFS 1 to 7 and institutionalization ($rs(935) = 0.43$; $p < 0.001$), and no significant relationship between CFS 7 to 9 and institutionalization ($rs(77) = -0.10$; $p = 0.399$).(102)

Kaplan-Meier survival curves for CFS frailty scores (Figure 2.2a), where CFS 1 to 4 are presented individually, and CFS 5 to 9 are presented together, in view of the relationship between CFS and 30-day mortality from visual inspection of the bar graphs. The log-rank test demonstrated a significant relationship between CFS and survival ($p < 0.001$).

ROC analysis (Figure 2.2b) for the prediction of 30-day mortality for the CFS and the NHFS, demonstrates that the CFS predicts 30-day mortality similarly to the NHFS (CFS AUC 0.631 (95% CI 0.572 to 0.690); NHFS AUC 0.631 (95% CI 0.571 to 0.690)).

Table 2.1 Patient demographic data, pre-admission residence, functional status and number of comorbidities categorised by Clinical Frailty Scale.

Characteristics	**CFS**								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Patients n (%)	37 (2.9)	112 (8.9)	222 (17.7)	236 (18.8)	227 (18.1)	249 (19.8)	156 (12.4)	14 (1.1)	2 (0.2)
Male n	9	37	76	76	63	60	39	3	2
Female n (%)	28 (75.7)	75 (67.0)	146 (65.8)	160 (67.8)	164 (72.2)	189 (75.9)	117 (75.0)	11 (78.6)	0 (0.0)
Mean age (95%CI)	76.95 (74.24, 79.65)	77.18 (75.56, 78.60)	81.80 (80.90,82.70)	85.53 (82.57, 84.50)	84.84 (83.82, 85.86)	86.47 (85.60, 87.35)	86.57 (85.45, 87.69)	88.36 (86.05, 90.66)	85.0 (-16.65, 186.65)
Mean AMTS (95%CI)	9.76 (9.57, 9.94)	9.32 (9.02, 9.61)	8.73 (8.46, 9.00)	8.02 (7.67, 8.37)	7.05 (6.64, 7.45)	4.86 (4.41, 5.32)	2.31 (0.31, 3.69)	2.0 (0.31, 3.69)	4.5 (-52.68, 61.88)
Mean NHFS (95%CI)	3.89 (3.55,4.23)	4.05 (3.86, 4.24)	4.52 (4.36, 4.67)	4.86 (4.71, 5.02)	5.23 (5.08, 5.39)	5.67 (5.53, 5.82)	6.03 (5.85, 6.20)	6.29 (5.43, 7.15)	8.0 (-4.71, 20.71)
Underweight,n (%)	5 (14.3)	5 (5.0)	14 (6.9)	21 (9.8)	30 (15.2)	33 (15.9)	23 (19.3)	2 (20.0)	0 (0.0)
Overweight, n (%)	8 (22.9)	40 (39.6)	67 (32.8)	80 (37.2)	62 (31.5)	52 (25.1)	18 (15.1)	1 (10.0)	0 (0.0)
Under or Overweight, n (%)	13 (37.1)	45 (44.6)	81 (39.7)	101 (47.0)	92 (46.7)	85 (41.1)	41 (34.5)	3 (30.0)	0 (0.0)

Characteristics	**CFS**								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Pre-admission residence									
Own home, n	37	111	217	223	205	183	87	6	2
Residential care,n	0	1	3	8	16	40	43	2	0
Nursing home, n	0	0	2	5	6	26	26	6	0
Pre admission mobility n (%)									
Freely Mobile	35 (94.6)	97 (86.6)	141 (63.5)	73 (30.9)	44 (19.4)	34 (13.7)	30 (19.2)	2 (14.3)	0 (0.0)
Mobile with 1 aid,	2 (5.4)	13 (11.6)	64 (28.8)	114 (48.3)	75 (33.0)	49 (19.7)	21 (13.5)	3 (21.4)	0 (0.0)
Mobile with 2 aids/frame	0 (0.0)	1 (0.9)	15 (6.8)	42 (17.8)	95 (41.9)	118 (47.4)	56 (35.9)	3 (21.4)	1 (50.0)
Some indoor no outdoor mobility	0 (0.0)	1 (0.9)	2 (0.9)	5 (2.1)	10 (4.4)	42 (16.9)	37 (23.7)	3 (21.4)	0 (0.0)

Characteristics	**CFS**								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
No functional mobility	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.4)	1 (0.4)	8 (5.1)	0 (0.0)	1 (50.0)
Needs assistance with ADLs, n (%)	0 (0.0)	3 (2.7)	13 (5.9)	39 (16.5)	61 (26.9)	90 (36.1)	84 (53.8)	6 (42.9)	1 (50.0)
Mean number of comorbidities per patient (95%CI)	1.19 (0.85, 1.53)	1.54 (1.30, 1.79)	2.10 (1.89, 2.31)	2.43 (2.19, 2.66)	3.07 (2.84, 3.30)	3.20 (2.99, 3.41)	3.35 (3.08, 3.61)	3.79 (2.58, 4.99)	1.50 (-4.85, 7.85)

Table 2.2 Frailty outcomes categorised by Clinical Frailty Scale Score. [1]Chi-sqauared test. [2]ANOVA with Levenes test for homogeneity and Welch test. [3]Spearmans correlation test.

	CFS									
	1 (n=37)	**2 (n=112)**	**3 (n=222)**	**4 (n=236)**	**5 (n=227)**	**6 (n=249)**	**7 (n=156)**	**8 (n=14)**	**9 (n=2)**	**P Value**
30-day mortality, n (%)	0 (0.0)	2 (1.8)	6 (2.7)	11 (4.7)	19 (8.4)	18 (7.2)	14 (9.0)	1 (7.1)	0 (0.0)	<0.001[1]
Presence of complications, n (%)	8 (21.6)	27 (24.1)	91 (41.0)	120 (50.8)	112 (49.3)	126 (50.6)	72 (46.2)	6 (42.9)	0 (0.0)	<0.001[1]
Home to institution, n (%)	2 (5.6)	9 (8.6)	56 (28.7)	100 (49.3)	99 (57.9)	102 (66.2)	59 (80.8)	3 (75.0)	1 (50.0)	0.427[3] <0.001[1]
Home to home, n (%)	34 (91.9)	96 (86.5)	139 (64.1)	103 (46.2)	72 (35.1)	52 (28.4)	14 (16.1)	1 (16.7)	1 (50.0)	
Mean Length of stay, days (95%CI)	9.03 (7.81, 10.25)	11.69 (10.67, 12.72)	15.17 (14.04, 16.30)	18.11 (16.51, 19.70)	18.12 (16.40, 19.85)	18.64 (16.92, 20.35)	18.28 (15.99, 20.58)	15.69 (10.08, 21.30)	9.00 (-3.71, 21.71)	<0.001[2]

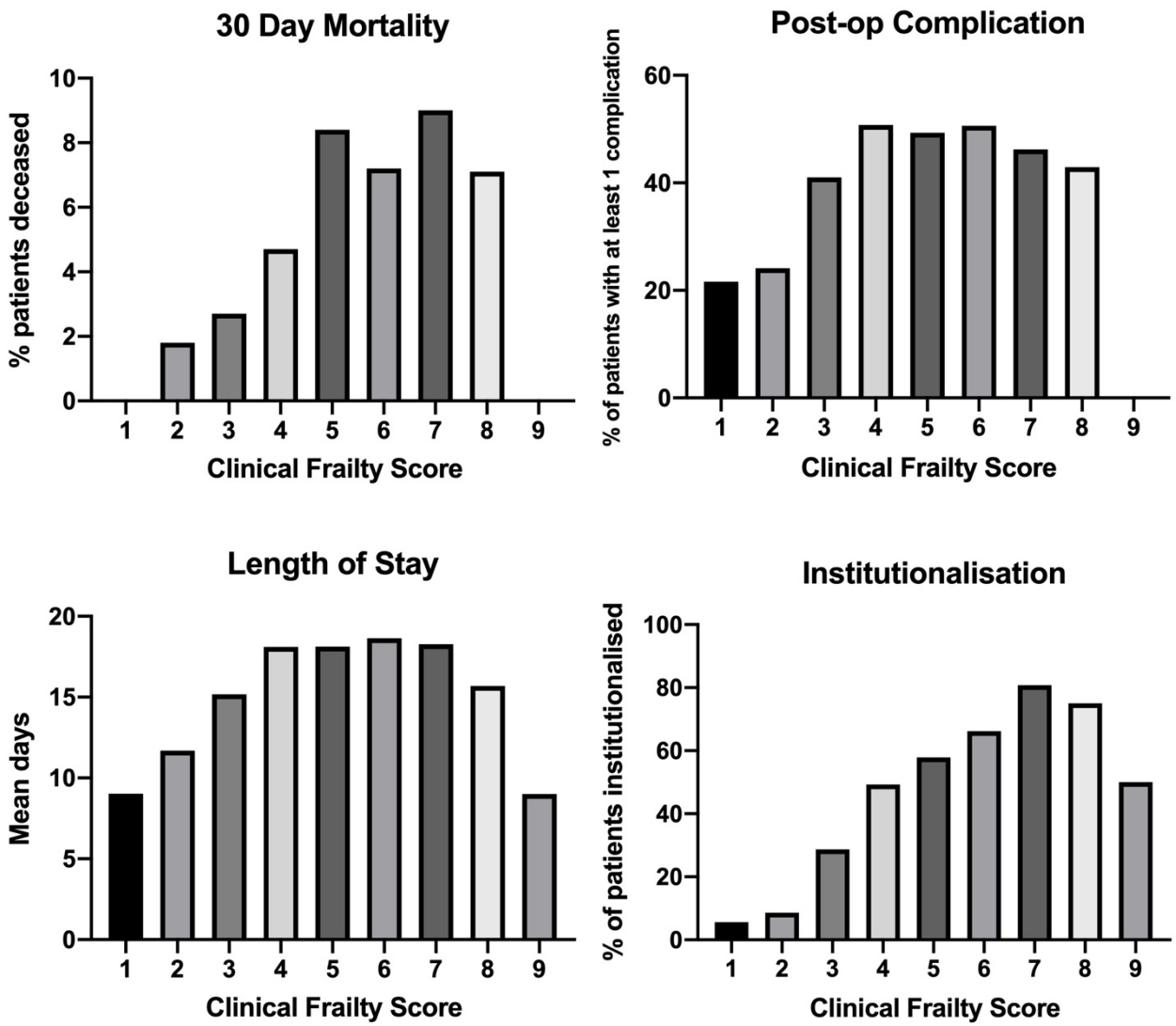


Figure 2.1 Bar graphs showing frailty outcomes stratified according to Clinical Frailty Scale Score. (top left) CFS and 30-day mortality, (top right) CFS and development of at least 1 post operative complication, (bottom left) CFS and mean length of stay, and (bottom right) CFS and new institutionalisation.

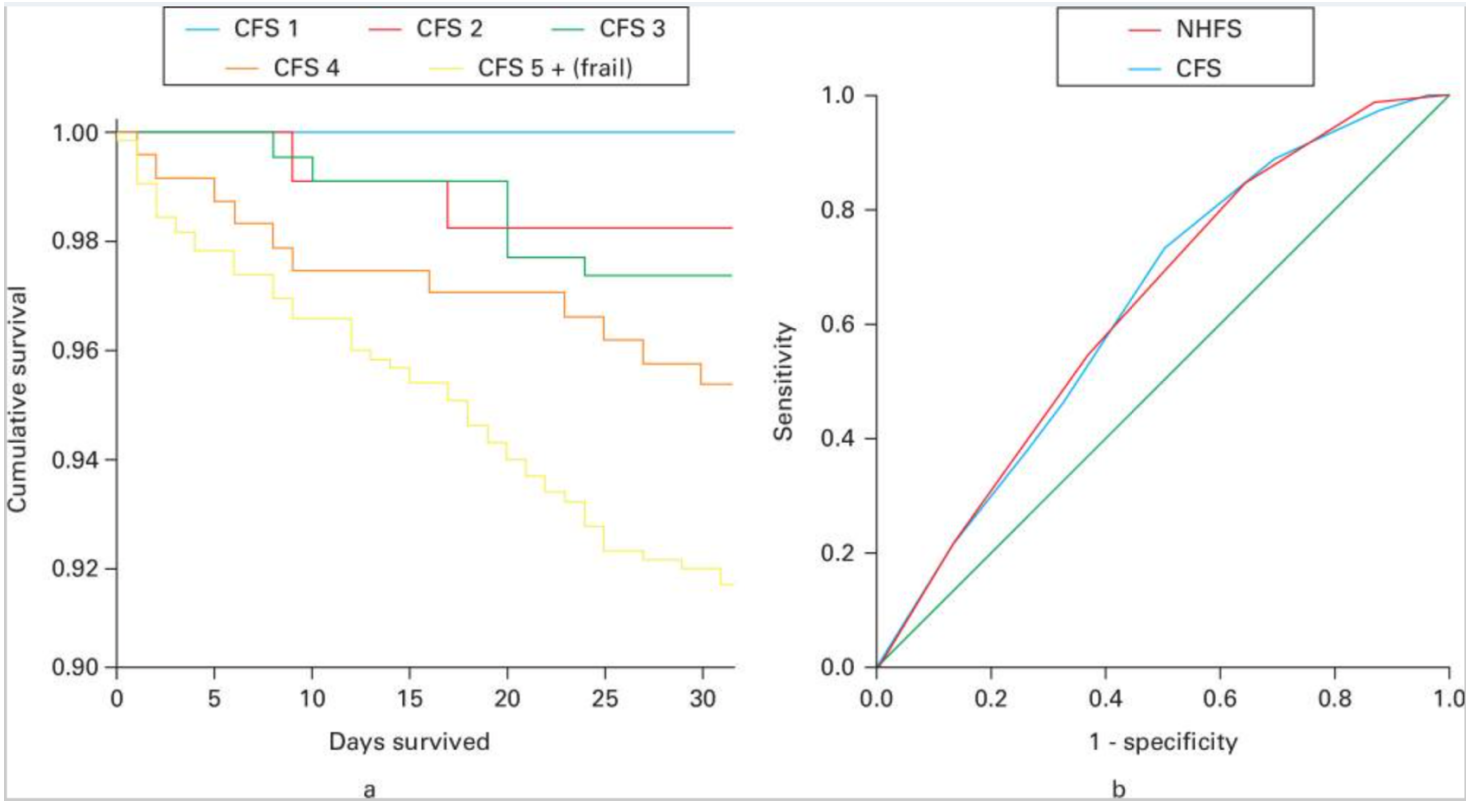


Figure 2.2. a Illustrates Kaplan-Meier survival curves for Clinical Frailty Scale (CFS) frailty scores, where CFS 1 to 4 are presented individually, and CFS 5 to 9 are presented together, in view of the relationship between CFS and 30-day mortality discussed previously. The log-rank test demonstrated a significant relationship between CFS and survival ($p < 0.001$). **b** Illustrates the Receiver Operating Curve (ROC) for the prediction of 30-day mortality for the CFS and the Nottingham Hip Fracture Score (NHFS). This demonstrates that the CFS predicts 30-day mortality similarly to the NHFS (CFS AUC 0.631 (95% CI 0.572 to 0.690); NHFS AUC 0.631 (95% CI 0.571 to 0.690)).

2.5 Discussion

Our study demonstrated a linear correlation between CFS 1 to 4 (non-frail patients) and surgical outcomes. It may be assumed that this linear relationship will continue or become steeper as the transition to increasing levels of frailty occurs. However, although our research indicates that patients with CFS scores ranging from 5 to 9 (moderate to severe frailty) have a poorer prognosis than those who are not frail. there is no correlation between higher frailty scores and mortality, postoperative complications, or length of stay (LOS) following hip fracture surgery. The exception is the institutionalisation, where there is a linear relationship between CFS 1 and 7. In conclusion, our results indicate that frail patients have a worse prognosis than those who are not frail, but we have been unable to distinguish disparities in prognosis for higher frailty levels using the CFS. Given the growing prominence of the CFS, it is essential to comprehend this limitation and understand why this may occur.

Frail patients have diminished physiological reserves, and as a result, their ability to 'bounce back' after injury may be diminished. There may be a physiological threshold above which outcomes do not change further. This may explain the shift from a linear to a nonlinear relationship between non-frail (CFS 1 to 4) and frail (CFS 5 to 9) mortality, postoperative complications, and LOS. With the linear progression of institutionalisation from CFS 1 to CFS 7, it is probable that the CFS is accurately measured in this cohort. The results of this investigation suggest that the CFS accurately quantifies frailty and therefore permits prediction of the probability of returning to baseline function (as measured by return to independent living). In the acute injury and perioperative setting, however, frail patients experience equally dismal outcomes regardless of their degree of frailty.

On the other hand, it is conceivable that the inability of the admitting clinicians to differentiate between the higher frailty grades explains the absence of an association between increasing frailty and increased complication rates. This may be the result of assessing the patient at the time of a severe injury, when a more complex history may

be difficult to ascertain, or a dearth of expertise. Recently, additional instruments have been introduced to facilitate more accurate assessment completion.

2.5.1 Strengths and Limitations

This is the first large-scale study investigating CFS as a predictor of outcomes following fragility fracture of the hip. The CFS is becoming part of routine data collection for hip fractures and often is collected by admitting teams. We believe these results to be robust, since the sample size was large, and the data were collected prospectively to a high level of completeness. The limitation of routine data collection processes meant other potential frailty outcomes such as incidence of delirium or residual mobility or functional deficit was not collected. The estimate for length of stay was for the acute hospital admission only and did not include time spent in rehabilitation wards and hospitals. This would have confounded the observed relationship between CFS and length of stay.

Even though this study demonstrates the importance of the principle of frailty measurement and the utility of the CFS tool for this purpose, the quantification of frailty remains a subject of substantial debate. There may be a need for more sophisticated methods of measuring frailty (such as using combinations of simple but valid biomarkers of organ or system integrity), and this should be investigated further and to explain why there is no difference in mortality, postoperative complications, and LOS between mildly, moderately, and severely frail patients.

3 Has changing established care pathways during the 1st wave of the Covid-19 pandemic led to an excess death rate in the fragility fracture population. Preliminary work

3.1 Overview

During the design of this thesis and precatory work the Covid-19 pandemic occurred. This had a profound impact on the health service in the United Kingdom. According to the Office for National Statistics in England and Wales, a total of 218 837 fatalities occurred between 1 March and 30 June 2020. Of these, 50 335 (or 23%) were attributed to COVID-19.(244) The number of daily fatalities was lower than the five-year average at the start of March 2020, potentially due to moderate winter influenza levels. By the conclusion of the initial week of April 2020, this value had more than doubled from the five-year mean, thereby illustrating the substantial influence of COVID-19 on all- cause mortality.

Given the reliance of this body of work on established and carefully controlled and reported standardised care pathways we studied the effect of changes associated with Covid-19 care on hip fracture care pathways in our institution and the impact this would likely have on subsequent work.

3.2 Introduction

Orthopaedic service delivery was fundamentally altered by the initial surge of the COVID-19 pandemic. Multiple modifications to established practice were mandated by the British Orthopaedic Association and government guidelines to maximise bed availability for patients inflicted with Covid-19, minimise the need for admission of elective patients, and prevent the spread of the virus. Elective operative practice was discontinued, trauma operative capacity was reduced to accommodate the expansion of intensive care bed capacity into operating rooms, and all grades of orthopaedic staff (including surgeons, medical personnel and allied healthcare professionals) were

reallocated to frontline emergency and intensive care. To increase hospital bed capacity, established evidence-based care pathways were modified to reduce length of stay whenever practicable and discharge thresholds. In the wake of a national lockdown, the government issued a directive to the public, advising them to work remotely and undertake essential travel only.

During the national lockdown there was a reduction in the general public's activity and hence a corresponding decline in the presentation of major and polytrauma activity. However, the number of patients presenting with fragility hip fractures did not cease. The World Health Organisation (WHO) declared in April 2020 that the elderly population is most susceptible to contracting COVID-19.(245) A recent study revealed that individuals aged 80 years and above have an age-related gradient of 7.8% increased mortality risk.(246)

Effect on hip fracture care

Excess deaths resulting from causes other than COVID-19 infection during the initial phase of the worldwide COVID-19 pandemic has been documented in studies.(247) Non-COVID-19 excess mortality is correlated with advancing age, with frailty and dementia accounting for the greatest increases in non-COVID-19 mortality. Fragility fracture patients are widely recognised to have a decreased prognosis, and these outcomes are care pathway dependent.

3.3 Methodology

We therefore conducted a longitudinal cohort study to quantify the excess mortality related to the pandemic following the modification of established care pathways in light of the first wave of infection in a UK hospital group with a major trauma centre and 1700 inpatients bed. The same institution to which this thesis study was being undertaken. This was accomplished by analysis of prospectively collected data for both the control and study group with data in both groups collected during patient admission as part of routine clinical care. The analysis of the control group was performed retrospectively as a pre-pandemic period was used as a comparator. We

wished to undertake an analysis of the effect of the pandemic on all fragility hip fractures with a subgroup analysis in those with fragility hip fracture. Including all fragility fractures would provide an overall insight to the change in mortality in frail patients at a local level since care for these patients is not nationally standardised for each injury subtype. In patients with fragility hip fracture this analysis could be taken into context with the scientifically established national care pathways which, are in effect at every hospital caring for these patients this makes the results of the study more generalisable.

Inclusion criteria for both groups were all patients, of all ages, admitted with an International Classification of Diseases (ICD)-10 code of S72 (fracture of femur), M80 (osteoporosis with current pathological fracture), M96 (intraoperative and post-procedural complications and disorders of post-procedural system, not elsewhere classified), W06 (fall from bed), W19 (unspecified fall) and Y79 (orthopaedic devices associated with adverse incidents). These ICD-10 codes were selected to capture patients who were likely presenting with fragility fractures and were identified from data entered for the national Hospital Episode Statistics (HES) submission. Patients who present with fragility fractures have significant medical comorbidities, are elderly and frail. This group of patients are the ones at greatest risk of both COVID-19 and any alterations to established evidence-based care pathways. Two time periods were selected to define groups: the study group were admitted during the first wave of the UK COVID-19 pandemic, between 1 March 2020 and 1 June 2020, and discharged by 30 June 2020; the control group were admitted between 1 March 2019 and 1 June 2019 and discharged by 30 June 2019. The pandemic was declared by the WHO on 11 March 2019, but in the days before this, there was an increase in the number of reported infections in the area served by our institution, so the study period was chosen from 1 March 2019. Excluded were all patients who did not have one of the ICD-10 codes above. Mortality was established using National Health Service Digital data.

Demographic and injury data collected included patient age and sex, the diagnosis, COVID-19 status (as identified in the medical records as ‘coronavirus SARS-CoV-2’-

positive patients or with a positive reverse transcriptase PCR), number of comorbidities and whether the patient had a surgical procedure (as identified through the procedure codes of the submitted HES data). COVID-19 testing was performed on admission for all patients and repeated every 3 days during inpatient stay; this was to facilitate and maintain safe distancing and isolation for COVID-19 contacts. Post-discharge testing was not performed as these patients were either discharged home or to an institution. Patients with hip fracture are not routinely followed up, and those patients with other fragility fractures were advised to not attend follow-up if they developed symptoms or had a positive test.

For the subgroup of patients with hip fracture, prospectively collected data were obtained from the institutional local hip fracture database. On this database, data for every adult patient admitted with a hip fracture are recorded using a modified version of the Standardised Audit of Hip Fractures in Europe data collection form. Data analysed included mechanism of injury, hip fracture type, body mass index (BMI), Abbreviated Mental Test Score (AMTS), mobility status (Fracture Mobility Score), Nottingham Hip Fracture Score (NHFS), haemoglobin concentration (as measured from the full blood count test) on admission and clinical frailty (as recorded on the Rockwood Clinical Frailty Scale). Data collected were confidential and managed in line with national data protection guidelines. Hospital outcome data were available for all patients and included type of surgery, mortality, requirement for invasive or non-invasive ventilation, length of stay and development of complications.

The data were extracted by a data analyst from our local database in July 2020 and ordered in Microsoft Excel (Redmond, Washington, USA) where numerical data comparison between groups was carried out using a students t-test assuming a two-tailed distribution with homoscedastic variance. For categorical data, where a 2×2 contingency table could be used, probability was calculated with the Fisher's exact test. For contingency tables greater than 2×2, the X^2 test was used. Statistical significance was set at $p<0.05$. Mortality was calculated from any death of any member of the cohort between 1 March and 29 June in the respective year. The Cox proportional-hazards model was used with STATA V.16.0 (StataCorp, Texas, USA)

statistical software package, to compare survival between groups and to calculate the HR where the COVID-19 first wave was the covariate. In addition, Student's t-test (assuming a two-tailed distribution with homoscedastic variance), Fisher's exact test and X^2 test were used to calculate probability.

3.4 Results

Demographic comparison between groups is shown in Table 3.1. The percentage of female patients and the number of hip fractures were equivalent between groups. Differences were noted with age and a lower percentage of patients receiving operative treatment during the COVID-19 pandemic.

Hip fractures were the most common diagnoses making up approximately 20% in each of the two comparative groups. These were selected for a more granular subgroup analysis. No differences were seen between subgroups for the variables selected other than prevalence of COVID-19 infection. Mortality was significantly higher in the cohort presenting with a fragility fracture during the COVID-19 pandemic period (Table 3.2) This was seen both in the COVID-19 period group as a whole and in the hip fracture subgroup analysis. It was also noted that the length of stay was significantly shorter for the COVID-19 period group and the COVID-19 period hip fracture subgroup.

Mortality of patients with fragility fracture without COVID-19 was significantly higher among pandemic period admissions (14.7%) than in the pre-pandemic cohort (10.2%) after adjusting for age and sex (HR=1.86; 95% CI 1.41 to 2.45; $p<0.001$) (Figure 3.1 and 3.2)

Table 3.3 shows the variable that may have influenced outcomes. The cohort of COVID-19-positive patients was significantly older than the COVID-19-negative patients in the same time period. In relation to excess deaths in patients without COVID-19, the COVID-19-negative patients had a significantly shorter length of stay,

lower frequency of operative treatment and less than expected ventilatory support than controls. There were no differences seen for sex or age between groups.

An analysis of patients with hip fracture revealed a mortality of 8.4% among 190 admissions in the pre-pandemic set, and of 15.48% among 168 pandemic admissions with no COVID-19 diagnosis, resulting in an HR=2.08 (95% CI 1.11 to 3.90; p=0.021). After further adjustment for clinical frailty scores, this became HR=2.15 (95% CI 1.15 to 4.04; p=0.0162).

Table 3.1 a. Descriptive characteristics of all fragility fracture patients presenting during the two study periods. **b.** Hip fracture subgroup analysis for the two study periods rPCR (reverse transcriptase polymer chain reaction), BMI (body mass index), AMTS (abbreviated mental test score), Hb (whole blood haemoglobin), RCFS (Rockwood Clinical Frailty Scale); [1]Student t-test assuming a 2-tailed distribution with homoscedastic variance. [2]Fisher exact test. [3]Chi-squared statistic calculated on a 2x3 contingency table.

a.

Factor	**COVID-19 period group (Admitted between 01/03/2020 - 01/06/2020) (n= 832)**	**Control period group (Admitted between 01/03/2019 - 01/06/2019) (n= 1014)**	**p value**
Mean age, years (± SD)	74.8 (± 20.0)	72.8 (± 21.4)	0.045[1]
Female, n (%)	492/832 (59.1%)	629/1014 (62.0%)	0.213[2]
rPCR COVID-19 positive, n (%)	104/832 (12.5%)	0/1014 (0%)	<0.00001[2]
Undergoing operative procedure, n (%)	70/832 (8.4%)	765/1014 (75.4%)	<0.00001[2]
Hip fracture, n (%)	180/832 (21.6%)	190/1014 (18.7%)	0.129[2]

b.

Factor	**COVID-19 period hip fracture subgroup (total: n= 180)**	**Control period hip fracture subgroup (total: n= 190)**	**p value**
Mean age, years (± SD)	83.2 (± 8.8)	81.9 (± 9.2)	0.183[1]
Female, n (%)	132/180 (73.3%)	139/190 (73.2%)	1.000[2]
rPCR Covid positive, n (%)	12/180 (6.7%)	0/190 (0%)	<0.0001[2]
Mean BMI, Kg/m^2 (± SD); n	24.2 (± 5.2); 159	23.2 (± 4.4); 169	0.070[1]

Mean AMTS, score (± SD); n	6.9 (± 3.5); 179	6.7 (± 3.9); 189	0.538[1]
Mobility status, n (available; %) • Freely mobile, no aids • Outside with aids • Unable to mobilise outside	n=175 61/175 (34.9%) 107/175 (61.1%) 7/175 (4.0%)	n=187 75/187 (40.1%) 96/187 (51.3%) 16/187 (8.6%)	0.076[3]
Mean Nottingham Hip fracture score (± SD); n	0.082 (±0.063); 172	0.081 (± 0.054); 181	0.838[1]
Anaemia on admission, Hb <12 g/L, n (%)	36/175 (20.6%)	45/182 (24.7%)	0.378[2]
Hip fracture type, n (%) • Undisplaced intracapsular • Displaced intracapsular • Intertrochanteric • Reverse oblique or subtrochanteric	14/180 (7.8%) 76/180 (42.2%) 77/180 (42.8%) 13/180 (7.2%)	16/190 (8.4%) 83/190 (43.7%) 79/190 (41.6%) 12/190 (6.3%)	0.851[2] 0.398[2] 0.834[2] 0.837[2]
RCFS, mean category from 1-9 (± SD); n	4.8 (± 1.6); 155	4.6 (± 1.8); 175	0.421[1]

Table 3.2 Outcomes for each group. **a**. Outcomes for the full cohort. **b**. Outcomes for the hip fracture subgroup. SD (Standard Deviation), HR (Hazard Ratio), n (number). [1]Kruskal-Wallis test.

a. Outcomes	**COVID-19 period group (n= 832)**	**Control group (n= 1014)**	**Hazard Ratio (HR) and p value**
Mortality, n (%)	150/832 (18.0%)	104/1014 (10.2%)	HR 1.86; 95%CI 1.41-2.45; p<0.0001
Length of stay, mean days (± SD); n	8.8 (± 8.9), 832	12.1 (14.3), 1014	p<0.001[1]

b. Outcomes	**COVID-19 period hip fracture sub-group (n=180)**	**Control hip fracture subgroup (n=190)**	**Hazard Ratio (HR) and p value**
Mortality, n (%)	30/180 (15.5%)	16/190 (8.4%)	HR=2.08; 95%CI 1.11-3.90 p=0.021
Mortality adjusted for clinical frailty, n (%)	30/180 (15.5%)	16/190 (8.4%)	HR=2.15; 95%CI 1.15-4.04 p=0.0162
Length of stay, mean days, (± SD); n	11.1 (± 5.5); 180	15.3 (± 7.4); 190	p<0.001[1]

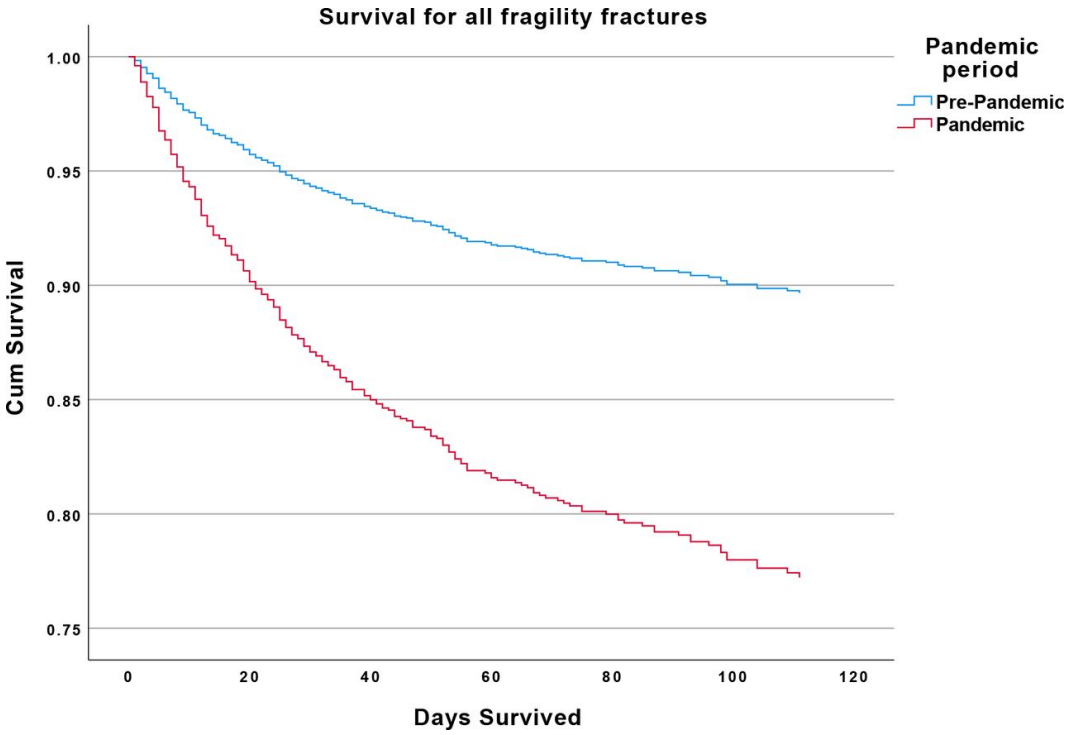


Figure 3.1 Reduced survival in non-COVID-19 fragility fractures. Kaplan-Meier survival plot. Red: COVID-19 pandemic (COVID-19 negative) patients with fragility fracture. Blue: pre-COVID-19 pandemic patients with fragility fracture.

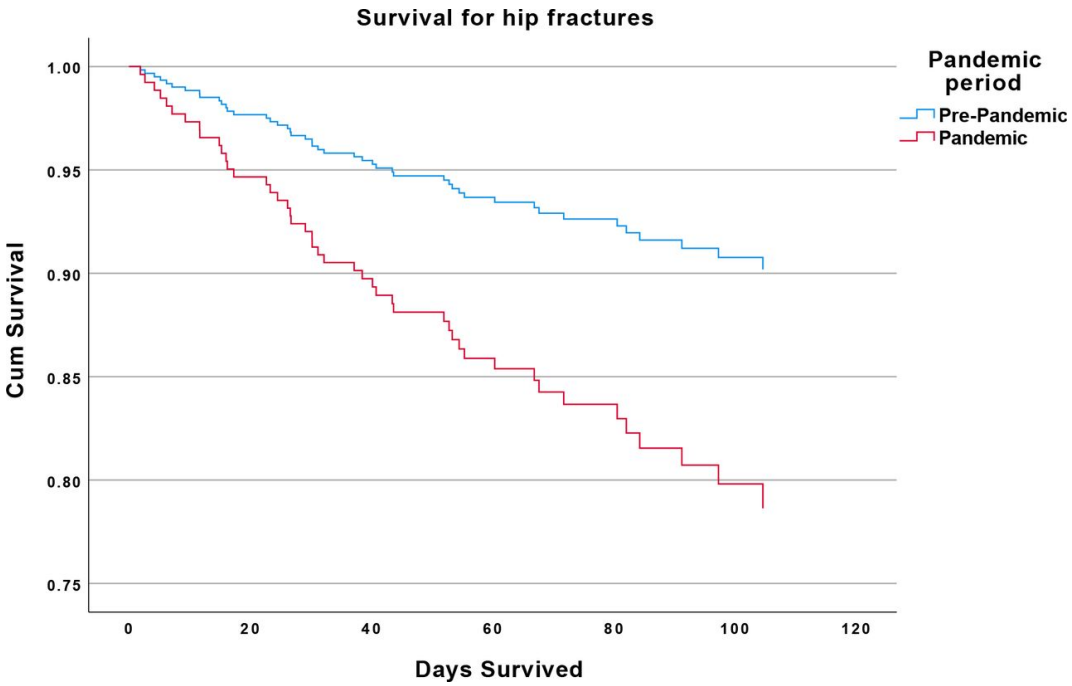


Figure 3.2. Reduced survival in non-COVID-19 fragility hip fractures. Kaplan-Meier survival plot. Red: COVID-19 pandemic (COVID-19 negative) patients with fragility hip fracture. Blue: pre-COVID-19 pandemic patients with fragility hip fracture.

Table 3.3 Complications, patient, and treatment factors by group. [1]2-tailed distribution with homoscedastic variance Student's t-test. [2]Fisher exact test. [3]Kruskal-Wallis test. [4] Chi-squared statistic calculated on a 2x3 contingency table. * Data was only available for 165 patients.

a.	COVID period group COVID-19 positive (total: n= 104)	COVID period group COVID-19 negative (total: n=728)	Control period group (total: n= 1014)	p value
Age on admission; mean years (±SD); n	81.0 (±11.9)	73.9 (±20.7)		0.001[1]
		73.9 (±20.7)	72.8 (± 21.4)	0.301[1]
Male sex; n (%)	47/104 (45.2%)	292/728 (40.1%)		0.338[2]
		292/728 (40.1%)	385/1014 (38.0%)	0.370[2]
Length of stay; mean days (± SD); n	14.8 (±10.6)	8.0 (±8.3)		<0.001[3]
		8.0 (±8.3)	12.1 (±14.3)	<0.001[3]
Operative treatment; n (%)	12/104 (11.5%)	58/728 (8.0%)		0.255[2]
		58/728 (8.0%)	765/1014 (75.4%)	<0.00001[2]
ARDS or sepsis, n (%)	5/104 (4.8%)	26/728 (3.6%)		0.576[2]
		26/728 (3.6%)	26/1014 (2.6%)	0.254[2]
Ventilation post procedure, n (%)	6/104 (5.8%)	24/728 (3.3%)		0.253[2]
		24/728 (3.3%)	54/1014 (5.3%)	0.046[2]

b.		COVID period hip fracture subgroup COVID-19 positive (Total: n= 12)	COVID period hip fracture subgroup COVID-19 negative (Total: n= 168)	Control hip fracture subgroup (Total: n= 190)	p value
Age on admission; mean years (±SD); n		82.5 (±6.3)	83.1 (±9.0)		0.818[1]
			83.1 (±9.0)	81.9 (± 9.2)	0.181[2]
Male sex; n (%)		4/12	44/168 (26.1%)		0.736[2]
			44/168 (26.1%)	51/190 (26.8)	0.905[2]
Length of stay; mean days (± SD); n		17.3 (±7.2)	10.7 (±5.15)		<0.001[3]
			10.7 (±5.15)	15.3 (± 7.4)	<0.001[3]
Hip fracture treatment.	Arthroplasty	8/12	68/165* (41.2%)	81/190 (42.6%)	0.227[4]
	Fixation	4/12	90/165* (54.6%)	103/190 (54.2%)	0.355[4]
	Non-Operative	0/12	7/165* (4.2%)	6/190 (3.2%)	0.587[4]
Pulmonary embolus or DVT		0/12	0/168	2/190 (1.1%)	
Pneumonia		3/12	14/168 (8.3%)	13/190 (6.8%)	0.081[4]
Urinary Tract Infection (UTI)		3/12	7/168 (4.2%)		0.021[2]
			7/168 (4.2%)	10/190 (5.3%)	0.804[2]
Cerebrovascular Accident (CVA)		0/12	0/168	0/190	

Myocardial Infarction (MI) or Acute Coronary Syndrome (ACS)	0/12	2/168 (1.2%)	0/190	
Dislocation or failure of fixation	0/12	0/168	1/190 (0.5%)	
Blood transfusion	2/12	32/168 (19.1%)	40/190 (21.1%)	0.856[4]
Renal failure	2/12	18/168 (10.7%)	22/190 (11.6%)	0.813[4]
Pressure ulcer	0/12	1/168 (0.6%)	1/190 (0.5%)	
Clostridium difficile	0/12	0/168	0/190	
BMI	24.7 (±3.5); 9	24.27 (±5.1);150		0.768[1]
		24.27 (±5.1);150	23.2 (± 4.4); 169	0.072[2]

3.5 Discussion

In this study, we report significantly higher mortality among patients with fragility fracture with no COVID-19-related diagnosis admitted during the COVID-19 pandemic period compared with patients admitted with the same ICD-10 codes to the same hospital in the same time period of the year 2019. We also report significantly shorter hospital length of stay during the months of the pandemic compared with the same period a year earlier. These results hold true after adjustment for potential confounders such as age and sex. A sub-analysis on femoral neck fractures where frailty indices and other clinical assessments were readily available showed a similar pattern while there was no difference in frailty between the pandemic and pre-pandemic cohorts.

Limitations of this study include potential variables that have not been controlled that may have influenced the effects that have been seen. Knowledge of the patients with fragility fracture who contracted COVID-19 in the community, who subsequently died within the period of assessment, is not known and may account for a proportion of the excess mortality. In addition, it is known that during the first wave period of investigation in this study, the institutional compliance with the best practice tariff was reduced. There is the potential that the redeployment of staff during the COVID-19 first wave, particularly the orthogeriatric support and physiotherapists, for patients with fragility fracture, may have had a negative influence on outcomes. However, specific data about the effect of redeployment as a variable are not available for this study.

Reports from the Office for National Statistics found that England had the highest overall relative excess mortality out of all the European countries compared during the first 6 months of 2020.(248) While none of the four UK nations had a peak mortality level as high as Spain or the worst-hit local areas of Spain and Italy, excess mortality was geographically widespread throughout the UK during the pandemic, whereas it was more geographically localised in most countries of Western Europe. Our data add to this picture by showing that excess mortality involves patients without

COVID-19 and areas of England with rates of infection below the national average. Achievement of best practice hip fracture tariff (249) at our institution during the pandemic fell from an average of 42% (March–June 2019) to 38% (March–June 2020) with a particular failure to achieve the delirium assessment, which fell from an average of 96% to 62% for the respective periods. In addition, there was a significant decrease in the number of patients with hip fracture who were not delirious after surgery, from 81% to 63%, respectively. This indicates breakdown of standard care pathways due to extrinsic pressures and provides a likely explanation for the excess mortality. As there are no agreed pathways or metrics for other fractures, it is impossible to prove, although probable that the same factors apply.

Hip fractures occurred at the same frequency during the COVID-19 period and the national lockdown, as before it, with no differences identified for age, sex, BMI, AMTS, mobility, NHFS, anaemia, fracture type or frailty between groups. Similar incidence pre-COVID-19 and post-COVID-19 with similar demographics allows a valid comparison to examine excess deaths.

Analysis of the COVID-19-negative subset of those presenting during the COVID-19 period compared with the control period does identify associations that may be relevant. While there were no differences identified between these groups for age, the development of Acute Respitatory Distress Syndrome (ARDS) or sepsis of the requirement for ventilatory support post-procedure, significant difference was noted in the COVID-19 period group (COVID-19-negative subset) for a shorter length of stay and reduced number of procedures. Both differences suggest that the standard care pathways were altered for fragility fractures, which is further supported by the observed fall in Best Practice Tarrif (BPT) achievement. A reduced length of stay is often considered a positive outcome. However, if the threshold for a safe discharge is altered, for instance in the face of a great need for more inpatient beds to manage a pandemic, there may be unintended consequences affecting mortality. A significant proportion of patients with fragility fractures require input from health and social care in the form of rehab services and residential homes. During the pandemic period, it was unknown regarding the virulence of spread of COVID-19 transmission within care

homes and limiting testing in this group of people was available; thus, this may have also influenced our mortality findings.

For those presenting with hip fractures during the COVID-19 period, who did not test positive for COVID-19, that no differences were seen for age, sex, BMI, treatment type (including non-operative treatment) and complications including: venous thromboembolism, urinary tract infection, stroke, myocardial infarction, failure of fixation or dislocation, transfusion, acute renal failure, pulmonary embolus or *Clostridium difficile* when compared with the control group suggests that understanding the reasons for the excess mortality is complex. Despite this, reduction in length of stay seems to be a significant factor in the hip fracture subgroup analysis, where changes to operative treatment frequency (as for the fragility fracture group as a whole) are not. It has been demonstrated that a COVID-19 infection independently causes an increased mortality compared with those presenting with hip fractures who do not contract COVID-19.(250-252)

During the COVID-19 period, only 12 patients tested positive for COVID-19. When compared with those presenting during the same time period, they were older and had a longer length of stay. Age, as a risk factor for developing COVID-19, is well established.(253)

During the COVID-19 pandemic period, services were subjected to changes in both personnel and patient pathways. Although one may assume that there would have been a reduction in staff due to an increase in sickness and testing positive for COVID-19, the measures put into place did not impact the staffing levels and actually improved the staffing levels. Although at the expense of the following, trainee doctors did not rotate to other specialties or units to maintain continuity of care, clinicians out of programme for research/experience and research nurses were brought back into clinical practice, doctors were placed onto megarotas and staff normally working in the elective setting were moved to the acute setting to improve patient flow. The change in the established evidence-based guidelines for patient care pathways is altered to help improve patient flow within the hospital, facilitate discharge and

improve capacity for the at time unknown effect of the pandemic. These were approved both regionally and nationally by subspecialty working groups.(254)

3.5.1 Strengths and Limitations

This study is one of the largest cohort studies in patients with fragility hip fracture investigating the excess mortality in COVID-19-negative patients during the first wave of the COVID-19 pandemic. Specific International Classification of Diseases (ICD)-10 codes allowed us to identify the same injury patterns over both groups and a comparative time matched cohort was used as a control. The effect of the provision of personal protective equipment and transmission between healthcare staff and patients was not known and these data were not available. The redeployment of support staff particularly physiotherapists and orthogeriatric staff for patients with fragility fracture may have had an influence on negative outcomes; however, specific redeployment data were not available for this study.

This study suggests that excess deaths are likely due to changing established, evidence-based care pathways, particularly regarding thresholds for safe discharge and length of stay. Excess mortality seen in this vulnerable population with fragility fracture is likely to be minimised by maintaining standards of care during further waves of the COVID-19 or future pandemics.

4 Functioning of Elder Muscle Understanding Recovery (FEMUR) study. Frailty and the hip fracture population.

4.1 Purpose

This chapter describes in detail the methods common to experimental chapters. This chapter also informs the background to the FEMUR study and explains the rational of choice of chosen outcome measures. Where a method is specific to an experimental chapter, it will be discussed in its own methods section.

4.2 Background

Core to the concept of frailty is sarcopenia. Physical activity regimes have been found to ameliorate frailty markers in older community dwelling individuals.(255) The efficacy of rehabilitation programmes following hip fracture in those who are very frail is equivocal. Cognitive impairment, poor mobility prior to fracture, and co-morbidities impacts the ability of patients to engage in these programmes effectively.(256)

Deep phenotyping is one way of identifying pathways associated with outcomes. In this study tissue from muscle biopsies were analysed to determine the concentration of metabolites and their intermediaries, the functioning of biochemical pathways, and the associated expression of genes within the muscle cell. (257-259) It has led to advancements in understanding of the cellular physiology of the muscles of elderly individuals in recent years. Few research studies have analysed muscle samples from individuals as frail as those who present with hip fractures or investigated the metabolic changes that occur in the muscle following injury in this population. Frail individuals are vulnerable, and frequently have moderate cognitive impairment. Recruitment and participation in musculoskeletal research studies is challenging and explains why this population is underrepresented in the literature. Little is known about the cellular physiology of the muscles of the extremely frail, and there is a dearth of data to guide the rational selection of novel treatment strategies for

sarcopenia. Cross-sectional studies on healthy elderly individuals and prospective studies attempt to replicate the muscle wasting seen in sarcopenia (using immobilisation or bed rest protocols). However, the results of these studies are not generalizable to frail patients.

We designed an unselected prospective cohort study of individuals with frailty, including those with cognitive impairment, as part of the overall study and a subset of the cohort. Those with dementia or severe cognitive impairment are typically the frailest and account for a significant proportion of patients admitted with a hip fracture. If these patients were excluded, we risked observing muscle changes associated with milder levels of frailty, which may not provide a complete picture. This is a widely held criticism and limitation of previous research in this field. In fact, if cognition is an essential determinant of 'frailty,' then these patients should be studied if results are to be generalizable to a larger population of older, frail adults and if interventions applicable to severe frailty states are to be developed in the future. Patient and public involvement input to increase participation among the target population has played a role in the development of the protocol and selection of outcome measures.

Higher surgery-induced inflammation was significantly related to worse muscle performance and fatigue in the first post-operative days as well as after more than one month following surgery.(260) The FEMUR study provides a unique opportunity to add to this existing data and extend understanding on how inflammatory markers in the blood relate to frailty outcomes.(261)

In summary, the research study aims to improve our understanding of the function, and molecular signalling of skeletal muscle of frail older individuals, including their relationship to patient-related outcome measures. In the longer term it is envisaged that innovations in the diagnosis and treatment of frailty can be achieved by translating findings from human physiology and metabolism research into the clinical setting.

4.2.1 Rational for choice of outcome measures.

4.2.1.1 Muscle mass assessment rationale for choice

The increased risk of radiation from CT scanning was felt to not appropriate in this cohort of patients. Also due to the Covid-19 pandemic this resource was in high demand for acutely unwell patients. It was also felt that logistically transferring patients from the ward to the scanner would impede patient recovery. MRI was not deemed appropriate either many of the patients admitted with hip fracture also have cognitive impairment and would need to remain still for up to an hour for an MRI scan. It was felt that they would not be able to do this leading to suboptimal images and a waste of resource and time. To assess muscle mass D3 creatine dilution method was chosen as many of the patients due to poor mobility would have indwelling catheters which would make urine collection more reliable. This analysis has been undertaken but falls outside the scope of this thesis.

Ultrasound assessment of vastus thickness was also deemed to be appropriate as a single measure to correlate this with D3-creatine muscle analysis to see if this would be an appropriate bed side alternative to measure muscle mass with no exposure to radiation.

4.2.1.2 Muscle strength assessment rationale for choice.

Extensive literature has already demonstrated the use of hand grip strength as a measure of muscle strength, this along with the working group guidelines utilising hand grip strength as one of the early measures to define patients with sarcopenia made this an obvious assessment tool to use for this study. A simple bed side assessment can be undertaken by any healthcare professional. From our own experience in previous studies in similar cohort of patients this assessment has been tolerated well.

4.2.1.3 Muscle performance assessment rationale for choice

The TUG test has been shown to be one of the tools to confirm a diagnosis of sarcopenia in different working group definitions. It has also been used in literature

for patients with hip fracture. Although the time to complete the test is used to define whether a person has sarcopenia or not it was felt that the ability to complete the test irrespective of the time may also provide useful information as a binary assessment or predictor of discharge back home. Since the physiotherapy teams undertake the rehabilitation of patients following surgery it was felt that this assessment would be best completed by them to ensure patient safety and consistency the test and form part of their rehabilitation.

4.2.1.4 Frailty assessment

The CFS has been widely adopted due to the needs of the acute frailty network by NICE and NHFD in the assessment of hip fracture patients and is deemed to be the most appropriate initial screening tool to identify and grade frail patients. Existing work has been shown to highlight that those are frail despite level of frailty have similar outcomes which warrants further investigation. It is a simple assessment already routinely completed by the orthopaedic teams on admission for all patients and is established within the institution. The age cut-off for inclusion in this study has been set as 65 years and older this is due to the CFS as an objective measure of frailty only being validated in those 65 years and older.

The GFI allows for a subjective classification of frailty this is deemed to be important with regards to follow up. Nearly all patients who sustain a hip fracture are not routinely followed following discharge. CFS is an objective comprehensive assessment of a patient on admission. It would be difficult to assess if frailty state changes objectively from admission to post-discharge, as CFS is not completed at any other time point in the care of these patients. Therefore, GFI this has been included as a measure to assess frailty following discharge to observe if the frailty state for patients changes due to this injury, and how muscle performance affect frailty state. We have used this in previous studies, and it has been tolerated well.

4.2.1.5 Global assessment of health-related quality of life

The EQ-5D was chosen as a global assessment of health-related quality of life. It is advocated by both NICE and NHFD as part of follow up and widely been documented in its use in hip fracture care. It can be completed by the patient and by a proxy which would be required in patients with cognitive impairment. It is part of the agreed core outcome set for hip fracture studies.(75)

4.2.1.6 Subjective and objective functional performance

The RMI has been used extensively in stroke patients with mobility problems and it is envisaged that patients with hip fracture will exhibit similar mobility problems primarily affecting one side of their body. A significant number of patients with hip fracture have cognitive impairment, similar to patients with stroke. The RMI which already established in practice at our institution seems a logical choice in the subjective assessment of muscle function and it has also been used by the research team in previous studies and has been tolerated well.

The literature has demonstrated use of the BADL as a form of measuring functional outcomes in patient with hip fracture. Along with this it has also been used at our institution in previous research studies and tolerated by the elderly cohort well. It provides an objective assessment of patient function, of which the data can be collected by caregivers or proxies where the majority of those who are frail will be in rehab or residential or nursing homes following discharge.

4.3 Methodology

4.3.1 Study management

4.3.1.1 Ethical and Human Tissue Act approval

Patients who are frail are a vulnerable cohort of individuals who often have cognitive impairments. For those (in the opinion of the surgical team) who are unable to provide informed consent, an appropriate consultee was included in the consent process. Consent to participate in this research study was entirely separate from consent to undergo surgical treatment. Therefore, when the patient was unable to consent for

themselves and an appropriate representative was not available to provide their opinion on the person's wishes, that patient would not enter the study, even if the surgeon and an Independent Mental Capacity Advocate make the decision to proceed with surgery.

Procedures used in this study have been chosen to be as easy for the patients as possible, to improve their acceptability to the target population. For example.

- Muscle biopsies have been scheduled to be taken whilst the patient is anaesthetised to reduce the anxiety and burden associated with having an additional invasive procedure.
- As all participants will have reduced mobility, the D3-creatine method to assess whole body muscle mass has been chosen as an alternative to DEXA scanning as the patient does not need to be moved with the former technique.
- As older patients routinely have an indwelling urinary catheter sited after hip surgery, which remains *in situ* for the first week after surgery, the inconvenience to the participant of providing a urine sample at specific time points is removed.
- Hand grip assessments and ultrasound measures of muscle thickness have been made previously in frail older patients by the research team and in literature and have been found to be highly acceptable to this patient group.

The study was not initiated before the protocol, informed consent forms and participant information sheets had received approval or favourable opinion from a Research Ethics Committee (REC). When a protocol amendment was made that requires REC approval, the changes in the protocol were not instituted until the amendment and revised informed consent forms and participant had been reviewed and received approval or favourable opinion from the REC. A protocol amendment intended to eliminate an apparent immediate hazard to participants or researchers may be implemented immediately provided that the REC was notified as soon as possible, and an approval was requested. Minor protocol amendments only for logistical or administrative changes may be implemented immediately; and the REC

was informed. Favourable opinion of the study was granted by London-Camden & Kings Cross Research Ethics Committee on 9th July 2020 (REC reference:20/LO/0841). Favourable opinion of the amendments to collect bone and serum tissue, was granted by London-Camden & Kings Cross Research Ethics Committee on 17th September 2021 (REC reference:20/LO/0841). Health Research Authority and Health and Care Research Wales Approval was granted on 14th July 2020.

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki, 1996; the principles of Good Clinical Practice and Good Laboratory Practice, the UK Department of Health Policy Framework for Health and Social Care, 2017 and in accordance with the Human Tissue Act, 2004

4.3.1.2 Screening and Recruitment

Patients admitted to our institution by the on call orthopaedic team were presented in the trauma multidisciplinary meeting where the history and examination findings were discussed, and management plans were formulated. Here most of the screening was undertaken to ensure possible participants met the inclusion and exclusion criteria prior to approaching potential participants. Any discrepancies or ambiguity in the history or presentation was clarified with the patients' clinical notes and with the patient or if appropriate consultee. Once the participant was deemed eligible to be recruited to the study, they were approached by a member of the research team. The study was explained in detail to them, and an information leaflet provided. Time was given to the patient to read the information and to decide if they wished to participate. A second visit was made to the patient, if the patient wished to take part in the study the consent forms were completed, and a unique identifier assigned. Initial questionnaires were then completed and surgical consent for tissue samples added to the surgical consent forms. If the participant lacked consent, then a consultee was sought usually this was the next of kin. The clinical team looking after the patient was also informed of the participants wish to be included into the research study. Patients included were entered onto a database where contact information was kept for follow up questionnaires to be completed along with a checklist of required tasks to be undertaken or omitted appropriate to the patient's consent obtained such

as obtaining single or bilateral muscle samples, bone samples, completion of hand grip strength an ultrasounds measurement, audit of notes on discharge etc.

4.3.1.3 Eligibility criteria

The eligibility criteria for inclusion into the FEMUR study is as follows.

Inclusion criteria

- Aged over 65 years (no upper age limit).
- Clinical frailty scale score (assessed as part of standard of care) ≥4.
- Fractured hip sustained following a fall, that requires surgery.
- Good understanding of spoken and written English language.
- Able to give informed consent, or ability to provide assent and availability of a legally acceptable surrogate to provide consent.
- Inclusion criteria for bone sample collection: undergoing hip replacement surgery.

Exclusion criteria

- Those who fell and sustained their hip fracture greater than twelve hours prior to hospitalisation.
- Those who have fallen and sustained their hip fracture whilst as an inpatient.
- Surgery carried out later than 96 hours after the fall.
- Chronic neurological, inflammatory, or musculoskeletal disorders which result in muscle wasting. E.g. multiple sclerosis, muscular dystrophy, rheumatoid arthritis, and stroke.
- Any co-morbidity which precludes hip surgery.
- For the study subset, those with compromised swallowing reflex which would prevent the participant from taking fluids orally will be excluded.
- Those taking diabetes medication.

4.3.1.4 Informed consent

The process for obtaining participant informed consent was in accordance with the research ethics committee (REC) guidance, and Good Clinical Practice (GCP). All

participants able to do so provided written informed consent. Where the patient did not have capacity to consent for themselves, their nominated personal representative ('Consultee') was contacted. This consultee was not a staff member or a paid professional.

Prior to seeking consent, the investigator explained the details of the study to the patient, and where indicated also to their consultee, and provided a participant information sheet, ensuring that those involved had sufficient time to consider participating or not. The investigator answered questions that the participant or their consultee had regarding the study. The decision regarding participation in the study was entirely voluntary. The investigator emphasized to the participant (and if indicated also to their consultee) that consent regarding study participation could be withdrawn at any time without penalty or affecting the quality or quantity of their future medical care, or loss of benefits to which the participant was otherwise entitled. In the event of any conflict between the consultee and the patient, the patient's wishes were upheld.

Capacity to provide consent was determined by a member of the patient's direct care team as part of the standard consent procedure carried out for the planned surgical treatment. In the absence of capacity, consent to participate in the research project was sought from a personal consultee accompanying the patient. This person was any individual involved in the care or welfare of the person in a non-professional capacity and was usually the next of kin.

The informed consent form, or consultee form, was signed and dated before the participant entered the trial, or underwent any interventions related to the study, and agreement was obtained from the patient prior to carrying out any post-operative study procedures. The participant and where appropriate also the consultee received a copy of the signed and dated forms and the original was retained in the study master file. A further copy was filed in the participant's medical notes and a signed and dated note made in the notes that consent was obtained for the study. If the participant's health or cognition deteriorated and agreement to post-operative study procedures

could no longer be obtained, these procedures were not undertaken. However, unless the patient (or their nominee) requested to withdraw from the study, we continued to collect patient-related outcome data from their medical notes.

Should there be any subsequent amendment to the final protocol, which might affect a participant's participation in the trial, continuing consent was obtained using an amended consent form which was to be signed by the participant or their representative. If the consent form was amended during the study, the investigator followed all applicable regulatory requirements pertaining to approval of the amended informed consent form by the REC and use of the amended form (including for ongoing participants).

4.3.1.5 Funding

This study was primarily funded by the NIHR Nottingham Biomedical Research Centre (BRC) musculoskeletal theme. An additional successful application was obtained for a major research grant from AOUK&I for the value of £7600. The grant is open to orthopaedic, veterinary, and maxillofacial surgeons, trainees and operating room personnel involved in trauma care. The grant was earmarked for lab consumables, gene cards, production of case report forms and postage and packaging for follow up questionnaires. The grant will was paid in three instalments spaced across the duration of the study, upon signing of the contract, production of report detailing the progression of the study and final approval of the report of the study was also completed.

4.3.2 Tissue samples

Each participant was assigned a study identity code number for use on the samples, consent forms and other study documents and the electronic database to maintain anonymity.

4.3.2.1 Muscle samples

A document summarising the patient pathway from anaesthesia to recovery had been disseminated to theatre personnel ahead of the start date of the study. Once the patient has been requested from theatre for their surgery, the theatre team contacted the research team. This provided ample time for the collection of the surgical equipment needed to perform the biopsies and collection of the liquid nitrogen from the laboratory. Following the completion of the World Health Organisation (WHO) surgical checklist the correct patient and surgical procedure, the participants study consent was confirmed (confirming fracture side biopsy or bilateral biopsy). After successful administration of effective anaesthesia, during the surgical approach to the hip, a muscle biopsy of vastus lateralis (75-150mg; size of a small pea) was taken from all recruited participants. The sample was taken with a size 10 scalpel and non-toothed forceps while the muscle was exposed deep to the epimysium. The location of the sample was at the inferior aspect of the wound and proximal portion of vastus lateralis. The patient's surgical team carried out the biopsy and operating staff passed this onto the research team in a surgical gallipot for immediate snap freezing in liquid nitrogen. Once completed the samples were taken to the laboratory, labelled, and stored in a dewar containing liquid nitrogen.

For patients undergoing additional biopsies of the non-fractured leg, this was completed prior to patient positioning for those undergoing hip replacement. This was deemed appropriate, as to perform a hemi-arthroplasty or total hip replacement the patient is required to be in a lateral position with the non-fractured leg in contact with the operating table. The lateral position impeded access to undertake the biopsy and therefore the only alternative would be to undertake the biopsy after the main surgical procedure. This was not done as it would introduce the surgical procedure as a confounding factor in the genes or proteins being expressed, in the muscle tissue of the non-fractured leg via a potential systemic response. Also, compression of the vastus lateralis from the weight of the patient may also cause a local change in the physiology. During fracture fixation surgery the biopsy of the nonfractured leg was undertaken at the time of fracture reduction. This was to reproduce as close as possible the time between the biopsies between the different types of surgeries.

A point 10-15cm superior to the middle of the upper border of the patella was noted. The location on the leg from which muscle was obtained was identified as a region 4-5cm lateral to this point. The skin was cleansed with 2% Chlorhexidine solution. A small stab type incision <1cm was made in the skin and soft tissue. Muscle samples were obtained from the vastus lateralis using the Bergstrom percutaneous muscle biopsy technique.(262) Using a 5mm Bergstrom biopsy needle (size corresponding to the diameter of the window located above the tip of the needle) was then introduced into the incision site, and firm pressure was applied until a 'give' sensation was felt. This indicated the passage of the needle through the tough fascia – a layer of connective tissue surrounding the muscle. The inner cutting cylinder was subsequently alternately released from and pushed in to the needle to cut adjacent muscle. This technique provides an easily reproducible method of acquiring muscle samples. When performed correctly, it was quick and caused little trauma.(263, 264) Scarring was minimal and there was no need for repeated biopsies.(265) Suction was applied to the biopsy needle while the sample is being cut, which increased the likelihood of obtaining a good yield of tissue though a fist pass technique minimising local trauma.(266) It has been demonstrated that serial needle muscle biopsy sampling where 2.5cm is left between biopsy sites does not elicit confounding changes in the muscle transcriptome when analysed by microarray technologies.(264) In this study repeated biopsies were not undertaken through a separate incision as the tissue yield was always sufficient through the initial incision and technique.

Following this the needle was snap frozen in liquid nitrogen. Closure was performed with steri-strips and pressure dressing with direct pressure applied for 5 minutes, over the incision site, (longer in those anticoagulated). The Bergström biopsy was undertaken by the same member of the research team in all recruited participants. To maintain consistency, accuracy and reduced potential technique learning curves. The initial 10 patients for this technique were not snap frozen, this was to ensure that an adequate yield of tissue was obtained. Once the supervision process was completed and the yield obtained was adequate, the remaining patients had their samples snap frozen.

Side effects from the needle biopsy may include bruising, local tenderness, and muscle soreness for 24-48hrs which was explained to the patient or appointed consultee through the consent process. Bruising was prevented by applying pressure following the biopsy and pain was managed through the effect of anaesthesia at the time of the procedure, and post-surgery pain relief as part of standardised care for all hip patients with hip fracture. All Bergström wounds were reviewed by both the research and surgical care team to monitor for complications.

The muscle samples were stored in labelled cryovials in liquid nitrogen and then transferred to a dewar containing liquid nitrogen. All samples were stored in the medical school in liquid nitrogen until analysis performed. Once the study related analysis had been completed, samples were then stored within the University's research tissue bank for use in future research (under Dr William Dunn- Licence Number 12265) following participant agreement. Where participants did not agree for future use of samples in research, samples were destroyed at the end of the study in accordance with the Human Tissue Act, 2004.

RNA extraction from the frozen muscle samples were taken place in laboratories within the School of Life Sciences. mRNA expression of 384 gene targets (spanning several cellular functions) were determined using polymerase-chain reaction (PCR) amplification and a custom designed Taqman® microfluidic gene card. Results were collated using Ingenuity™ Pathway Analysis software (Qiagen) and used to predict functional changes in the muscle.

The Bergstrom biopsy technique was used to perform the muscle biopsy of vastus lateralis of the non-fractured leg. This is through a small (less than 1cm incision) after anaesthesia but prior to hip surgery. Standardisation of the technique was undertaken. Initial training in this technique involved 1-1 demonstrations from an experienced staff member who has performed this procedure on participants in previous studies conducted within our unit. This was followed by direct supervision of the technique on the first 10 patients recruited to the study via a checklist. This was

to ensure adequate technique and obtaining satisfactory quality and quantity of tissue for analysis. Following the supervision period competency was assessed and awarded by the experienced staff member. The tissue samples were inspected for yield obtained by an experience staff member proficient in the processing and mRNA extraction. All samples were deemed to be more than adequate with tissue available for further analysis to be used in future studies if the participants gave consent.

4.3.2.2 Serum

Prior to anaesthetic induction, a 15ml blood sample was obtained from participants and collected into three sterile evacuated tubes (Vaccutainer^{TM}) from the intravenous canula inserted by the anaesthetist (direct care team) as part of routine procedure. This blood sample was labelled with the participants identification code and transferred to the colleagues from the research team for processing and subsequent analysis for concentration of circulating inflammatory markers. The use of existing intravenous access to collect the sample has been chosen to reduce the burden to the patient and avoid them undergoing additional venepuncture procedures. Samples were processed by the research team by centrifuge to obtain serum, this was then transferred to labelled cryovials in 2ml aliquots and stored in -80° freezer (appendix 8.3.1) The serum samples were then sent for proteomic analysis in two batches to Olink in Uppsala Sweden

4.3.2.3 Bone

For participants who had consented to have bone samples taken for architectural analysis. This was taken at the time of surgery in patients who had undergone hemi-arthroplasty or total hip replacement. The femoral head of the fractured leg was excised with care by the surgical team and handed to the research team where it was placed in formalin and stored.

Once ready for processing the femoral head was marked and cut using a bone saw. Three regions of interest was investigated related to the trabecular within the femoral neck (i) subfovea (ii) superior femoral head (iii) inferior femoral head involving primary

tensile and compressive groups. Micro CT scanning was undertaken of samples 125mm^3 after which architectural analysis of the samples will be undertaken by NRecon and CTAn computer software. This was correlated with patient frailty and patient outcomes. This analysis whilst complete is beyond the scope of this thesis.

4.3.2.4 US Vastus thickness

Muscle thickness of the *vastus lateralis* at the mid-point between the greater trochanter and the midpoint of the lateral border of the patella, was assessed on one occasion postoperatively on both legs using B-mode ultrasonography (Mylab 25; Esaote Biomedica, Genova, Italy), with a 50 mm, 7.5 MHz, linear-array probe. This non-invasive technique was carried out at the patient's bedside by a single researcher. It required a hand-held transducer to be placed longitudinally to the thigh, lightly rested on the skin surface. Ultrasound coupling gel was applied to the skin to permit transition of the ultrasound waves which also acts as a lubricant, promoting smooth movement of the transducer on the skin surface. Once a clear image along the mid-sagittal axis of the *vastus lateralis* has been obtained, the anterior and posterior border of the vastus lateralis was identified and marked. The central vastus point was then marked and at this point the image was taken and stored electronically onto the computer hard drive (intrinsic to the ultrasound machine) for later analysis using the manufacturer's software.

This was repeated twice more, obtaining 3 images for each leg. Scans were identified by the participants' unique study identifier and transferred via an encrypted USB memory stick to a study-specific database held on a password protected University of Nottingham server. The technique was successfully used by members of the current research team in previous studies recruiting frail, older participants. The ultrasound equipment is owned and maintained by the University of Nottingham. The timing of the ultrasound is coordinated with the patient's wishes, avoiding mealtimes, drug rounds, physiotherapy sessions and within the first week following surgery.

Ultrasound training began with pre-reading of the ultrasound protocol as part of the FEMUR study protocol. This was followed by demonstration by the senior lab

technician and supervisor. Once familiar in using the ultrasound machine, practice and development of skills was enhanced over three weeks by completing ultrasound assessment of vastus lateralis in five healthy individuals of varying, ages, sex and body composition on multiple occasions. Once confidence was built in the technique, one complete set of measurements of vastus lateralis volume and thickness, was completed ten different times on each leg, on a single volunteer. This was to ensure competent and accurate repetition of technique and measurements. An analysis of the coefficient of variation was performed. Right vastus lateralis thickness (2%) Left vastus lateralis thickness (5%), Right vastus lateralis muscle volume (3%) and Left vastus lateralis muscle volume (2%). A value of 5% or less was deemed to demonstrate competence in performing ultrasound measurement.

4.3.2.5 Hand grip strength

Grip strength was measured on one occasion in both hands at a convenient time for the ward and the patient in the first week after surgery. Grip strength was measured using a university calibrated handheld dynamometer (Jamar Hydraulic hand dynamometer Serial number 20170410188, Performance health supply, Inc, Cedarburg, WI) The hand-grip dynamometer was adjusted so that the upper, adjustable bar sat comfortably at the second joint of the fingers when the fixed bar lay in the palm of the hand. The arm was placed in a comfortable position with the elbow flexed to 90 degrees and the dynamometer was supported underneath, so that the patient did not have to take the weight of the equipment. The participant was then asked to squeeze the bars together as hard as they could, and the grip strength was recorded. The dynamometer was taken out of the hand and 2 minutes rest given before the dynamometer was repositioned and the reading repeated. Three measurements were made on each hand with the best reading of the three being taken as the maximum hand grip value. The presence of arthritis or other disability which could affect the hand grip reading was recorded, along with hand dominance. The test was performed with the patient in the seated position with feet flat on the floor. If the test was not performed in this position due to the patient being unable to sit out, then it was performed in bed, with the bed adjusted so it was in a semi-seated position.

4.3.2.6 Timed up and go test.

For those who regain sufficient mobility, the TUG test was carried out prior to discharge (median 8 days after surgery), by a member of the physiotherapy team. The patient is seated in a chair with an arm rest (to assist the rise to standing), and a mark, three metres away from the chair, was made. They were asked to stand, walk to the mark, turn, walk back to the chair and sit back down. Walking aids, such as a frame or a stick could be used during the test. The time taken to complete the procedure was recorded. Those who were not mobile, or those with cognitive impairments and unable to follow the instructions, were not asked to perform this test. The test was performed by selected physiotherapists trained in performing the test. This was for patient safety when mobilising, to prevent further falls and complications. The physiotherapists performing the test were limited to a select group to aid consistency and accuracy. Initially the test was offered and attempted at 7 days post-surgery. If unable to complete, it was offered once more prior to discharge. The date of the test, time taken, and completion will be noted in the patient records and data set.

4.3.2.7 Case report forms

Case report forms (CRF) were completed on admission, on day-7 following surgery, and at day-90 post discharge. The admission CRFs contained the GFI, the BADL and the RMI. The day-7 and day-90 CRF constituted the same components of the admission CRF but included the EQ-5D. The CRFs was completed with questions being asked of the patient by a member of the care team, or by an individual from the research team who had been delegated the task by the clinical chief investigator. At 90 days post-surgery a CRF was sent to the patient, or to their care setting if the patient is not independently living. Completion of the questionnaire was by the patient with assistance from carers as required and was returned to the research team in a stamped addressed envelope which was provided. Alternatively, the questionnaire was completed via the telephone this was to reduce the potential risk of transmission of the SARS-Cov-19 during the global pandemic which occurred during

the time of the study. The patient's and or carer's wishes regarding follow-up was determined prior to discharge.

The GFI was used on admission to assess degree of frailty prior to the fracture. This assessment tool is comprised of 15 questions across 8 domains; mobility, vision, hearing, nutrition, co-morbidity, cognition, psychosocial and physical fitness. All questions form the basis of the history taking carried out at admission and would not represent an additional burden for the patient or care team. Questions are scored as 1 or 0 and summated to give a frailty index with a higher score indicating greater frailty. The BADL questionnaire was completed on admission to assess degree of independence prior to the fracture. This questionnaire is comprised of 10 questions covering activities of daily living (e.g. bathing, dressing, feeding), mobility and continence, and its completion on admission forms part of routine patient care. The BADL is scored out of 100, with a higher score indicating greater independence. The RMI questionnaire is comprised of 15 questions relating to the individual's ability to carry out a range of movements from turning over in bed to fast walking. Questions are scored as yes (1) or no (0), with a maximum score of 15, and a higher score indicating greater mobility. The EQ-5D was completed to assess the patient's current health.

The CFS score was also documented, this is routinely collected on patient admission. It is calculated after a comprehensive history and consultation with family and other healthcare professionals.

Upon discharge an audit of the patients' notes were performed to document baseline medications and any additional ones prescribed. Length of stay, post-operative complications and treatments, pre- and post-surgical blood results which are routine in their care. The data was used in the associative analysis with the phenotype markers from the muscle samples. The data was compared with anonymised data from patient not included in the study to ascertain if the study cohort was representative of the wider population of patients having surgical treatment for patients with hip fractures.

Post operative complications were audited to cover a range of medical and surgical areas common in frail patients. These complications were already part of our routine data collection for all hip fracture patients. They included 18 different complications following surgery which included, Pulmonary embolus (PE), pneumonia, urinary tract infection (UTI), cerebrovascular accident (CVA), myocardial infarction (MI)/ acute coronary syndrome (ACS), Deep vein thrombosis (DVT), acute delirium, acute kidney injury (AKI), post-operative anaemia, blood transfusion, dislocation, failure of fixation, wound problems, surgical site infection, reoperation within 120 days, pressure ulcer, *C.difficile* infection, and inpatient mortality. Acute inpatient length of stay was also observed. As this study was completed during the Covid-19 pandemic it was felt this was both appropriate due to the research burden and generalisable to other institutions.

30-day mortality was observed as this forms part of the data collection for the NHFD and would allow us to benchmark the data from this study with current national data. We also observed 6-month mortality as a surrogate for mortality through frailty associated decline. Due to the significant 1-year mortality associated with this injury, there would be significant loss to follow up of a significant number of participants based upon the initial target sample size. Therefore, the use of 6-month mortality would help to balance the follow up response rate and sample size. Other factors unrelated to acute functional decline following hip fracture may also be a confounding factor to 1 year mortality.

4.3.3 Statistical analysis

This is a primarily descriptive study, not testing any intervention or related hypothesis to be able to carry out a power calculation. Sample size was based upon the number of patients presenting to the institute at which the study was undertaken from previous studies and local databases. The proposed sample size was obtained to represent the number of patients that is expected to be required to obtain a representative sample of a frail older cohort. With a sample size of 200, the margin of

error is +/-7% (confidence interval 95%), with the margin of error increasing to +/-10% (confidence interval 95%) in a subset of 100 people.

We assumed no priori relationship between the different data variables. Initial analysis included descriptive statistics of the data including the mean, standard deviation, skewness, kurtosis, and range. Distribution of the data was analysed visually through histograms and skewness z test to inform and guide the statistical test required with any transformation of the data if applicable.

Spearmans and Pearsons correlation tests have been undertaken to demonstrate monotonic and linear relationships as appropriate. Pearsons was used for normally distributed, or interval ratio and Spearmans was used for ordinal and nominal data. Paired t-tests were completed for analysis of EQ5D-Index and health, RMI, GFI and BADL. Independent t-test have been undertaken of the mean data when comparing two groups. Levens test for homogeneity was completed to ensure equality of variance. If data demonstrated no equality of variance, the equality of variance 'not assumed' t-test, degrees of freedom and p values have been stated.

The ANOVA test was used to compare the means for three or more groups. If homogeneity of variance was satisfied Tukey HSD post-hoc analysis was undertaken to ascertain differences between groups. If homogeneity of variance was not met, a Welch test was completed with subsequent post-hoc testing with Games-Howells analysis.

Kaplan-Meier survival curve was used to analyse survival with log rank statistical test, for non-parametric variables. Logistic regression was undertaken to observe the effects of CFS, grip strength according to EWGSOP2 guidelines, and ability to perform timed up and go test on the likelihood that patients admitted with a hip fracture would be discharged back to their own home. Initially univariate analysis was completed to identify important covariates significance level was set at p value <0.05. Hosmer and Lemeshow test were undertaken as a test of model fit. Survival modelling was undertaken with Cox regression. Variables included subjective and objective measures

of quality of life, muscle function, and performance. From these specific metrics which demonstrated statistical significance via univariate analysis were included. Modelling was undertaken using a backward elimination stepwise approach. Chi-square goodness of fit was undertaken for model fit.

ROC analysis to ascertain the sensitivity and specificity of measurers of muscle function to infer the predictive value of binary outcomes such as discharge home and mortality. For continuous data Youden's statistic has been used to demonstrate a cut-off value for the maximum sensitivity and specificity for the greatest predictive value. In addition, specific statistical tests have been undertaken for the gene expression analysis which are described in Chapter 6.

5 Functioning of Elder Muscle Understanding Recovery (FEMUR) Cohort; the association between subjective and objective measures of quality-of-life, muscle function and performance, with frailty outcomes in hip fracture.

5.1 Chapter Overview

This chapter contains a description of the FEMUR cohort and details the pertinent findings of the subjective and objective measures of quality of life, muscle function, and muscle performance in relation the participants clinical frailty. We begin with the screening and reasons for exclusion of patients from the study. Patient demographics, comorbidities, admission details, mechanism and classification of injury, blood results and surgical data are also discussed. Overall patient reported outcome data, muscle strength and performance metrics are presented. Data is also stratified according to clinical frailty scale score. To contextualise the biological measurements, it is important to understand patient functional capacity. Outcomes can be assessed objectively with clinician reported scores or measurements or subjectively with patient reported scores. To guide the analysis of the gene expression of the muscle tissue, it is important to understand both the subjective and objective measures of muscle function and health in patients within our cohort who have sustained a hip fracture. This allows a meaningful interpretation of muscle gene and pathway expression, and how they relate to clinical outcomes, performance, and function. Specifically, phenotyping of the population with related relevant functional scores and measures allows the cohort to be dichotomised appropriately and biological measures compared in valid analysis.

5.2 Screening

The FEMUR study recruitment period was 11/01/21 – 19/12/22. A screening log was kept. A summary of the patients included are in Figure 5.1. In this period a total of 1529 patients were admitted to Queens Medical Centre with a hip fracture. Due to staffing resources and logistical issues 1120 patients were not approached to be recruited to the FEMUR study. Therefore 409 patients were eligible to be recruited and screened. Following application of the inclusion and exclusion criteria, 139 patients were recruited to the study. In Chapter 6 involving gene expression analysis 80 patients who consented for bilateral vastus lateralis muscle biopsy had their samples analysed. In Chapter 7 involving inflammatory marker proteomic analysis 59 patients had serum markers analysed, the proteomic analysis was an amendment to the FEMUR study to investigate the systemic role of inflammation in hip fracture outcomes, hence the lower sample number.

5.3 Patient cohort

5.3.1 Patient demographics & comorbidities

The patient demographics can be seen in Table 5.1. The mean age of patients was 86.36 years, with 76% of those recruited being female. The mean body mass index for patients was 23.87Kg/m^2. All patients were white ethnicity with 83.5% living in their own home. 38.8% were freely mobile without any walking aids, approximately 25% walked with 1 aid and 30% with 2 aids or a frame prior to the fracture. The majority of patient 46.8% had between 3-5 medical comorbidities. For brevity the top ten most frequent comorbidities are shown in the table 5.1

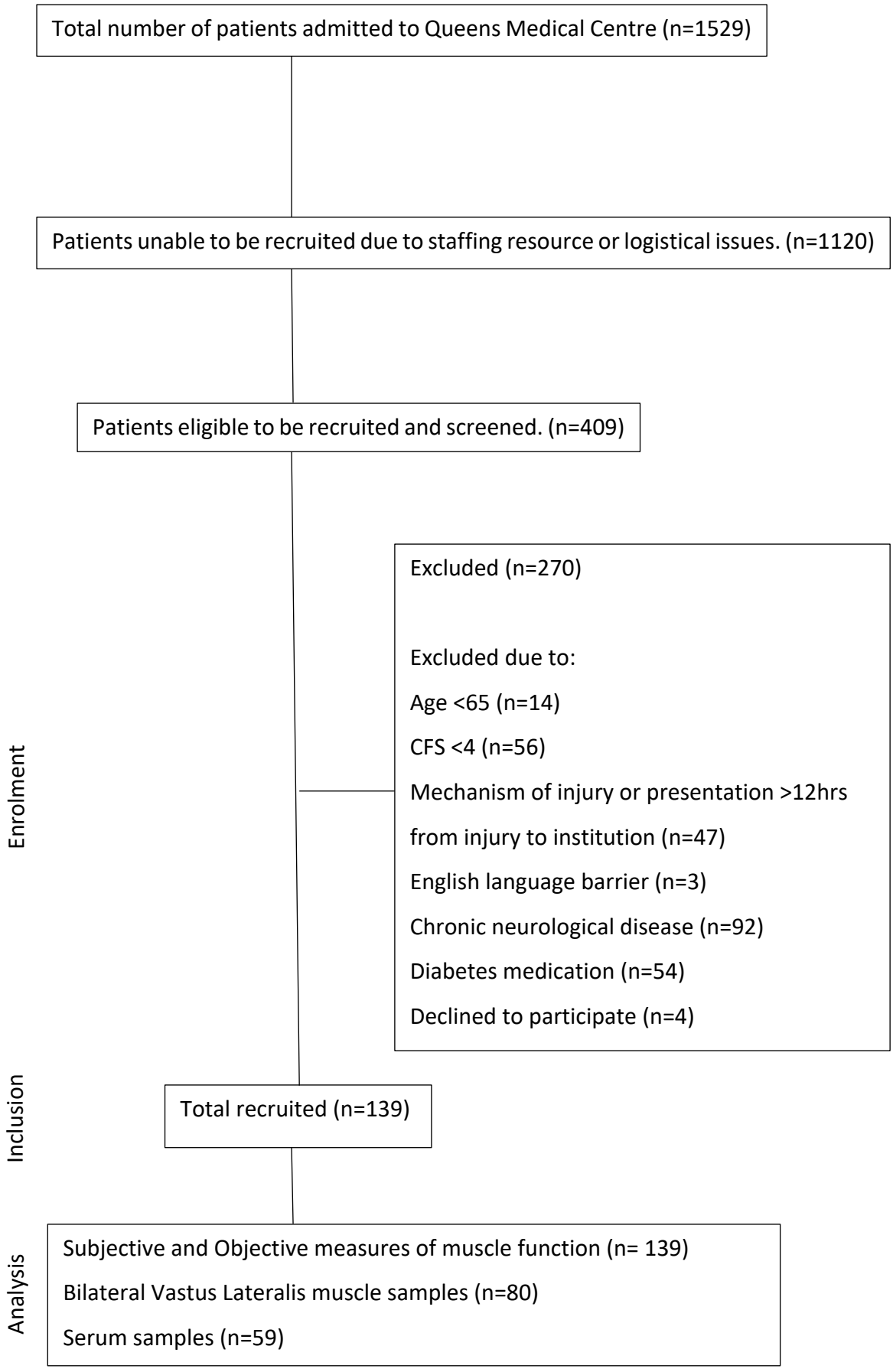


Figure 5.1 CONSORT flowchart of FEMUR cohort, participant recruitment.

Table 5.1 Patient demographics and comorbidities for all patients recruited to the FEMUR study.

Demographic	n	(%)
Mean Age (years)	86.36 (SD 6.744)	
Sex		
Male	33	23.7
Female	106	76.3
Mean BMI (kg/m^2)	23.87 (SD 5.0445)	
Ethnicity		
White	28	20.1
White British	110	79.1
White Irish	1	0.7
Residential status		
Home	116	83.5
Residential Home	13	9.4
Nursing Home	10	7.2
Pre-Injury mobility		
Freely mobile without aids	54	38.8
Walks with 1 aid	35	25.2
Walks with 2 aids or frame	42	30.2
Indoor mobility but never goes outdoors without help	8	5.8
Top ten comorbidities		
AF	34	24.5
Blindness/visual impairment	34	24.5
CKD	37	26.6
CVA/TIA	26	18.7
Dementia/cognitive impairment	37	26.6
Hypertension	84	60.4
Malignancy	20	14.4

Demographic	n	(%)
Osteoarthritis	30	21.6
Ex or Current smoker	35	25.2
Osteoporosis	22	15.8
Total comorbidities		
0 – 2	30	21.6
3 - 5	65	46.8
6 - 8	32	23.0
>9	12	8.6

5.3.2 Admission details, mechanisms & classification of injury

The admission details mechanism and classification of injury are shown in table 5.2. The mean time spent in the emergency department for all patients recruited to the FEMUR study was approximately 528 minutes. Many patients had a Nottingham hip fracture score between 4-6 which equates to a thirty-day mortality between 4-11%. Clinical frailty scale score ranged from 4-7 with no patient recruited with a frailty score of 8 or 9. The most common concurrent illness at the time of presentation with a hip fracture was a chest infection which occurred in 6.5% of patients. The mean abbreviated mental test score was 7.19. Most patients sustained a hip fracture due to a mechanical cause or a trip, this was followed by an unwitnessed fall. Two thirds of the patients sustained an intracapsular hip fracture.

Table 5.2. Admission details, mechanism and classification of injury.

Admission details, mechanism and classification of injury	n	%
Mean time spent in the emergency department (min)	528.11 (SD 277.366)	
NHFS Score (% risk of 30-day mortality)		
3 (3%)	8	5.8
4 (4%)	39	28.1
5 (7%)	40	28.8
6 (11%)	33	23.7
7 (16%)	19	13.7
CFS		
4	39	28.1
5	32	23.0
6	39	28.1
7	29	20.9
Concurrent illness at time of presentation		
Acute delirium	6	4.3
Chest infection	9	6.5
Urine infection	2	1.4
Heart failure	6	4.3
Uncontrolled dysrhythmia	4	2.9
Chest injury/rib fracture	0	0
Pubic rami fracture	1	0.7
Proximal humerus fracture	4	2.9
Distal radius fracture	4	2.9
Head injury	2	1.4
Spinal fracture	2	1.4
Mean AMTS on admission	7.19 (SD 3.447)	

Admission details, mechanism and classification of injury	n	%
Mechanism of injury		
Mechanical / trip	74	53.2
Syncopal / dizzy	11	7.9
Loss of balance	13	9.4
Weakness / legs giving way	10	7.2
Unwitnessed	31	22.3
Hip fracture type		
Intracapsular	88	63.3
Extracapsular	51	36.7
AO hip fracture classification		
31A1.2	10	7.2
31A1.3	6	4.3
31A2.2	6	4.3
31A2.3	12	8.6
31A3.1	1	0.7
31A3.2	2	1.4
31A3.3	7	5
31B1.1	14	10.1
31B1.2	2	1.4
31B1.3	39	28.1
31B2.1	27	19.4
31B2.3	2	1.4
31B3	7	5
32A1(a)	1	0.7
32A2(a)	3	2.2

5.3.3 Blood results and inflammatory markers

Blood results from admission, pre-surgery, and day 1 post-surgery are shown in table 5.3. There is a drop from admission haemoglobin to day 1 post-surgery demonstrating blood loss and there is also a reduction in the mean number of circulating platelets. It

appears kidney function through observing the eGFR remains stable but decreases slightly during in patient stay over the first 24-48hours.

Table 5.3 Admission, pre-surgery and day 1 post-surgery blood results for all patients recruited to the FEMUR study.

	Admission (±SD)	Pre-Surgery (±SD)	Post-Surgery (±SD)
Mean Haemoglobin g/L	124.95 (18.634)	114.73 (19.345)	105.43 (16.302)
Mean Haematocrit L/L	0.384 (0.614)	0.353 (0.057)	0.325 (0.050)
Mean Platelets $x10^9$/L	256.79 (122.440)	219.32 (108.866)	217.29 (94.025)
Mean eGFR mL/min/1.73m^2	64.86 (17.915)	66.62 (19.181)	62.17 (19.700)
Mean Parathyroid hormone ng/L			91.04 (62.448)
Mean Thyroid stimulating hormone mU/L			1.56 (1.202)
Mean Vitamin D nmol/L			53.33 (31.088)

5.4 Frailty cohorts

5.4.1 Patient demographics

Observing the FEMUR cohort in table 5.4 the only statistically significant difference between frailty groups was mean BMI, residential status and pre-injury mobility.

Table 5.4 Demographics of frailty cohorts, *ANOVA (F-statistic), $^{+}$Pearsons Chi Squared test

		CFS 4 n=39 (95%CI)	CFS 5 n=32 (95%CI)	CFS 6 n=39 (95%CI)	CFS 7 n=29 (95%CI)	P value
Mean Age Years		83.62 (81.52, 85.71)	86.50 (83.90, 89.10)	88.54 (86.76, 90.31)	86.97 (84.22, 89.71)	0.12 (3.812)*
Mean BMI Kg/m^2		26.459 (24.703, 28.215)	23.369 (21.479, 25.259)	23.359 (22.088, 24.631)	21.525 (19.836, 23.214)	<0.001 (6.347)*
Mean number of comorbidities		3.56 (2.80, 4.33)	5.22 (4.13, 6.31)	5.05 (4.39, 5.71)	4.52 (3.72, 5.32)	0.15 (3.606)*
Sex	Male	12	8	11	2	0.108^{+}
	Female	27	24	28	27	
Ethnicity	White	7	5	10	6	
	White British	32	27	29	22	0.530^{+}
	White Irish	0	0	0	1	
Residential status	Home	39	32	2	13	
	Residential Home	0	0	6	7	<0.001^{+}
	Nursing Home	0	0	1	9	

	CFS 4 n=39 (95%CI)	CFS 5 n=32 (95%CI)	CFS 6 n=39 (95%CI)	CFS 7 n=29 (95%CI)	P value
Pre-Injury mobility					
Freely mobile without aids	25	12	9	9	
Walks with 1 aid	11	8	10	6	<0.001[+]
Walks with 2 aids / frame	2	13	18	9	
Indoor mobility but never goes outdoors without help	1	0	2	5	
Top ten comorbidities					
AF	3	10	15	6	
Blindness/visual impairment	9	10	10	5	
CKD	11	10	12	4	
CVA/TIA	4	6	9	7	
Dementia/cognitive impairment	0	4	9	24	
Hypertension	26	18	24	16	
Malignancy	3	8	6	3	
Osteoarthritis	5	12	9	4	
Ex/Current smoker	8	12	10	5	
Osteoporosis	3	4	8	7	

5.5 Aims and objectives.

The aim is to evaluate the subjective and objective measures of function in frail patients who have sustained a hip fracture, before and after their injury, and how these relate to frailty outcomes (post operative complications, institutionalisation or discharge home, and mortality). The objectives have been defined following review of the literature and both primary research and agreed outcome measures such as the core outcome set which has been discussed in Chapter 1.

Objectives

1. To evaluate global health assessment at day 7 and day 90 following hip fracture using the EQ-5D.
2. To evaluate the pre-injury, day 7 and day 90 subjective measures of frailty and muscle function through the GFI and RMI respectively.
3. To evaluate the pre-injury, day 7 and day 90 objective measures of muscle function through the BADL, hand grip strength and timed up and go test.
4. To correlate these subjective and objective measures of muscle function with the CFS.
5. To understand the relationships between the subjective and objective measure of muscle function.
6. To evaluate the subjective and objective measure of function with frailty outcomes including post-operative complications, institutionalisation or discharge home and mortality.

5.6 Methodology

Patients were recruited as part of the FEMUR study as previously mentioned in chapter 2. Our institution collects routine data on all hip fracture patients using the Standardised Audit of Hip Fracture in Europe (SAHFE) collection form. Data was collected independently by trained audit staff who administer the local database according to national data protection standards. Additional data required for the study was collected by members of the research team following appropriate consent.

Global assessment, subjective measures of muscle function, and one objective measure of muscle function were obtained through case report forms. The first case report form contained the GFI, RMI and BADL questionnaires which was completed following the consent process to obtain the pre-injury level of function. This was undertaken jointly by the patient and a member of the research team. If the patient was not able to complete this due to cognitive impairment the consultee was substituted. A second case report form containing the GFI, RMI, BADL, and EQ-5D was then completed on day 7 following hip fracture surgery with the patient or member of the nursing team caring for the patient. The patient or consultee was then given a preference for the day 90 questionnaire to be either completed by post or telephone. As the study took place during the Covid-19 pandemic most patients and consultees elected to complete the questionnaire over the telephone to reduce the transmission risk of Covid-19. At day 90, patients or their consultee completed the same case report form containing the GFI, RMI, BADL and EQ-5D.

The EQ-5D scores from five domains were converted to an index value which we refer to as the EQ5D-index, each representing a unique health state. This was used in the analysis to provide a global assessment of function. The visual analogue scale component of the EQ5D we have referred to as EQ5D-health, which was also recorded and is a global measure of health rather than a unique health state. The GFI, RMI and BADL total scores were used in the analysis. Scores were all collated on a password protected Microsoft Excel Spreadsheet and stored in encrypted format according to general data protection regulation. Anonymised patient data was transferred to SPSS Statistics (version 29.0.1.0 for Mac) for statistical analysis.

The remaining objective measures of muscle function were collected by members of the research team. Hand grip strength was measured in kilograms using a calibrated handheld dynamometer on day 7, the technique has been previously discussed in chapter 2. The best hand grip strength overall was used for analysis. The hand grip strength was also classified into normal or low according to EWGSOP2 sarcopenia cut-offs for both male and female patients.

The TUG test was performed on day 7 following hip fracture surgery. To maintain strict safety during mobility this was completed by a member of the physiotherapy team. Patients were permitted to use walking aids and if the physiotherapist deemed the patient to be unsafe or not ready to undertake the assessment it was delayed until the patient could attempt it safely. If the patient was not able to perform the test during their entire admission and if the patient attempted the TUG but was unable to complete the TUG this was recorded as 'unable to complete TUG'. If the TUG was not assessed this was recorded as 'missed'. If the TUG was completed the time in seconds was recorded in the patients' clinical notes. If a patient declined to undertake the assessment this was also recorded. Upon audit of the notes prior to discharge a member of the research team recorded the TUG test assessment and the day which it was performed.

5.6.1 Statistical analysis

Statistical analysis was undertaken, and this has been described in Chapter 2.

5.6.2 Missing data analysis

The data has been analysed as a complete dataset with the assumption that data is missing at random and therefore there are no inherent biases introduced by this approach.(267) This has some inherent weaknesses which could be addressed with sensitivity analysis, or imputation however this is beyond the scope of this PhD. However, due to the size of the data set this is a reasonable assumption.

5.7 Results

In this chapter the focus is on the subjective and objective measures of quality of life, muscle function and performance and their relationship with the CFS. The results presented in this chapter reflect this. Detailed statistical analysis and graphical representation of data, of the relationships between the subjective and objective measures in all patients irrespective of frailty has been undertaken. These results are in appendix 9.12.

5.7.1 The relationship between CFS and EQ5D

There is a negative correlation between CFS and EQ5D-index and health. This was a weak correlation for EQ5D-index and moderate correlation for EQ5D-health. (Table 5.5)

Figures 5.2 & 5.3 show patients with a lower frailty score have a greater EQ5D-index and EQ5D health at both day 7 and day 90. As the frailty score increases there is a decline in the EQ5D scores. Patients with a CFS 7 demonstrate similar scores in EQ5D-index at day 7 and 90. Overlap of the confidence intervals in EQ5D-health suggests there may be no difference in scores within frailty groups.

ANOVA testing revealed a statistically significant difference in mean EQ5D scores between frailty groups confirming the visual interpretation (Table 5.6). Post-hoc testing (Table 5.7) showed significant statistical differences between CFS 4 and 7 in all EQ5D scores. There is a statistically significant difference between CFS 4 and 6 in EQ5D-health at day 7 and day 90, and EQ5D-index at day 7. CFS 5 and 7 show a significant statistical difference in EQ5D-index at day 90 and EQ5D-Health at day 7. This demonstrates at the extreme ends of frailty the pre-frail and severely frail, there are significant differences in quality of life at day 7 and day 90. At the points of transition between frailty groups the differences are non-significant. There are no statistically significant differences in EQ5D-scores between two adjacent frailty groups.

Paired t-test (Table 5.8) confirms a statistically significant improvement in EQ5D-Index at day 90 when compared with day 7 within frailty groups CFS 4, 5, and 6. There was no statistical improvement in CFS 7. There was no statistically significant improvement in EQ5D-health scores for any of the frailty groups.

5.7.2 The relationship between CFS and GFI

Spearmans correlation demonstrated a significant positive correlation between CFS and GFI. This was moderate at pre-injury and day 90 and weak at day 7 (Table 5.5).

Figure 5.4 shows the mean GFI to increase as the CFS increases. There is overlap of the confidence intervals between pre-injury day 7 and day 90 which may suggest no statistically significant difference in change of GFI score within frailty groups.

Between group analysis using ANOVA showed a statistically significant difference in GFI score across CFS groups, as shown in Table 5.6. Generally, as the CFS increases the mean GFI increases. Post-hoc testing shown in Table 4.4 revealed most differences lie in the pre-injury and day 90 GFI. The pre-injury GFI was statistically significantly different between CFS 4 and CFS 6 and 7, and CFS 5 and CFS 7. There was no statistically significant difference between two adjacent clinical frailty scores. The day 7 GFI demonstrated a statistically significant difference between CFS 4 and CFS 6 only. At day 90 There was a statistically significant difference between CFS 7 and all other CFS groups. There was also a significant difference between CFS 4 and CFS 5. This would suggest that the difference between CFS 5 and 6 is very narrow and would be supported by the fact that there is no statistically significant difference in GFI scores at any time point between CFS 5 and 6

For patients in CFS 4 paired t-test demonstrated a statistically significant increase in GFI from pre-injury to day 7, which then decreased at day 90. However, there was no statistically significant change when comparing pre-injury to day 90 GFI. This would suggest the patient returned to their pre-injury level of frailty (Table 5.9)

In CFS 5 there was no statistically significant change in GFI score suggesting no change in level of frailty. In CFS 6 and 7 there appears to be no overall change from pre-injury to day 90. However, there is a statistically significant change in GFI score from day 7 to day 90, in CFS 6 this is a decrease but CFS 7 an increase.

5.7.3 The relationship between CFS and RMI

Spearmans correlation showed a statistically significant negative correlation between CFS and RMI. This was strong for pre-injury, and moderate for day 7 and 90 (Table 5.5).

Figure 5.5 shows as the CFS increases the RMI decreases. The pre-injury RMI is the greatest amongst all frailty groups, and the day 7 is the lowest. At day 90 the RMI does not appear to return to pre-injury level except for possibly CFS 6. In CFS 7 there does not appear to be much improvement from the day 7 RMI.

Analysis with ANOVA reveals a statistically significant difference between the mean RMI scores at all three time points. As the CFS increases the RMI decreases (Table 5.6). Post-hoc analysis shows no statistically significant difference between CFS 6 and 7 in pre-injury RMI. There is no statistically significant difference between CFS 4 and 5 and CFS 5 and 6 at day 7. At day 90 the only statistically significant difference is shown at the extreme ends of frailty between CFS 4 and 7 and CFS 5 and 7 (Table 5.7).

Paired t-test showed patients in all frailty groups had a significant drop from pre-injury to day 7 in their RMI scores. All patients demonstrated an improvement in their RMI scores from day 7 to day 90 except those with a CFS 7. Only patients who had a CFS 6 returned to their pre-injury RMI score (Table 5.9).

5.7.4 The relationship between CFS and BADL

Spearmans correlation demonstrated a statistically significant negative correlation between CFS and BADL. This was strong with pre-injury and moderate with day 7 and day 90 BADL (Table 5.5).

Figure 5.6 shows patients with a lower CFS have a greater BADL at all time points. It also demonstrates that the BADL does not return to pre-injury levels in all frailty groups apart from CFS 6 where there is overlap of the confidence intervals. At CFS 7

there is no improvement in BADL from day 7 to 90. CFS 5 and CFS 6 also demonstrate similar BADL scores.

ANOVA testing between frailty groups shows mean pre-injury, day 7 and day 90 BADL is statistically significantly decreasing with increasing frailty (Table 5.6). Post-hoc analysis shows no statistically significant difference in CFS 4 and CFS 5 in pre-injury BADL, no statistically significant difference between CFS 5 and CFS 6 at day 7 BADL. At day 90 BADL the statistically significant differences are between CFS 4 and 7, CFS 5 and 7 and CFS 6 and 7, with no differences between CFS 4 5 and 6 (Table 5.7).

Paired t-test shows for all frailty groups there is a statistically significant drop in BADL from pre-injury to day 7. CFS groups 4, 5, and 6 although do not return to their original pre-injury BADL level they do improve from their day 7 BADL scores. CFS 7 do not improve in their BADL scores from day 7 (Table 5.9).

5.7.5 The relationship between CFS and VLT

Spearmans correlation demonstrated a significant weak negative correlation between CFS and both VLT-F and VLT-NF (Table 5.10).

Figure 5.7 demonstrates that the mean vastus lateralis thickness is greater in the fractured limb when compared to the non-fractured limb. The graph also shows as CFS increases the thickness of the muscle decreases.
ANOVA demonstrated a statistically significant difference between VLT-F and VLTNF thickness and frailty groups. Post-hoc analysis however revealed the only difference to be between CFS 4 and CFS 7 at the extreme ends of frailty (Table 5.6 and 5.7)

5.7.6 The relationship between CFS and TUG

Spearmans correlation shows a moderately negative corelation between CFS and ability to perform TUG test and Pearsons correlation demonstrates a moderately positive correlation with TUG time. (Table 5.10)

Figure 5.8 shows as the CFS increases the TUG time increases. Overlapping of the confidence intervals suggests adjacent frailty groups may show no statistically significant difference except at the extreme ends of the frailty scale. ANOVA with post-hoc analysis confirms as the frailty score increases the TUG time increases. The statistically significant differences occur between CFS 4 and 6, 4 and 7, and 5 and 7. There is no statistically significant difference between adjacent frailty groups (Tables 5.6 and 5.7). An association between CFS and ability to perform the TUG test was also observed. $X^2(3)=23.470$, $p<.001$ Table 5.11

5.7.7 The relationship between CFS and HGS

Spearman's correlation shows a significant moderate negative correlation between CFS and HGS and a moderate positive correlation with low grip strength according to EWGSOP2 criteria (Table 5.10).

ANOVA demonstrated a statistically significant difference between CFS and HGS, with increasing CFS there is a decrease in HGS Table 5.7 and Figure 5.9. Post-hoc testing showed the majority of the significant difference were between CFS 7 and the remaining frailty groups. There was also a statistically significant difference between CFS 4 and 6 Table 5.7.

5.7.8 Measures of function and relationship with frailty outcomes

5.7.8.1 Post operative complications

In our cohort we investigated 18 different complications following surgery. These included, PE, pneumonia, urinary tract infection (UTI), cerebrovascular accident (CVA), myocardial infarction (MI)/ acute coronary syndrome (ACS), DVT, acute delirium, acute kidney injury, post-operative anaemia, blood transfusion, dislocation, failure of fixation, wound problems, surgical site infection, reoperation within 120 days, pressure ulcer, *C.difficile* infection, and inpatient mortality. There were 28 patients who did not develop a complication and 111 developed one or more complication. The top 5 complications were post-operative anaemia, acute kidney injury, acute delirium, pneumonia, and the requirement of a blood transfusion We observed the

total number of comorbidities per patient figure and dichotomised into two groups patients with no complications and patients with 1 complication or more. Spearmans correlation demonstrated no statistical significant correlation between the development of 1 post operative complications and CFS, subjective and objective measures of muscle function (Tables 5.12 and 5.13) (Figure 5.10).

5.7.8.2 Discharge home

We first observed the residential status of the patients (Table 5.14), 116 patients lived at home, 13 in a residential home and 10 from a nursing home. No patient from a residential or nursing home was discharged to their own residence. Of the 116 patients from their own home 71 returned, 30 went to rehabilitation units and 5 became permanently institutionalised on discharge, the remaining 10 patients died as inpatients.

When observing patients who came from their own home, Spearmans correlation showed statistically significant correlations in patients who were discharged home with EQ5D at day 7, pre-injury and day 7 RMI, day 7 BADL, CFS, low grip strength (EQWGSOP2), ability to do TUG test, and TUG time (Table 5.15).

Binary logistic regression

To predict successful discharge home for patients admitted from their own home binary logistic regression analysis was undertaken. Objective measures were used since these were performed by the same group of individuals limiting variation and excluding changes in patient cognitive state as a confounding factor in subjective measures of muscle performance.

Only patients admitted from their own home were included in the modelling and the 10 patients who died as inpatients were excluded from the analysis as they were not classified as returning home or institutionalised. The assumptions for binary logistic regression were followed where the dependent variable was on a dichotomous scale the options being discharge home or not home. One continuous variable (day 7 BADL) and three categorical variables (CFS, HGS according to EWGSOP2 definitions and

ability to perform the TUG test). There was independence of observations, however Box Tidwell test (1962) showed there was not a linear relationship between the continuous variable, the day 7 BADL and the logit transformation of the dependent variable. Therefore, this had to be removed from the modelling, leaving only the three categorical variables.

The logistic regression was performed to ascertain the effects of CFS, grip strength according to EWGSOP2 guidelines and ability to perform timed up and go test on the likelihood that patients admitted with a hip fracture would be discharged back to their own home. The logistic regression model was statistically significant $x^2(5) = 37.894$, $p<.001$. The model explained 44.6% (Nagelkerke R^2) of the variance in being discharged home and correctly classified 78.7% of cases. Only the ability to perform the TUG test was associated with an increased likelihood of being discharged back home 13.195 times greater than those unable to complete the test (Table 5.16)

ROC analysis

The ability to perform the TUG test at day 7 post hip fracture surgery is a good measure to predict discharge back to home in patients admitted from their own home (AUC 0.788, [95%C.I. 0.690, 0.885]) p<.001. When observing the day 7 BADL this had a better predictive value than the ability to perform the TUG test (AUC 0.807, [95%C.I. 0.717, 0.896]). The maximum value for Youden's J statistic shows a cut off value of 47.50 for the BADL score would provide a 77.8% specificity and 73.7% sensitivity (Table 5.17 and Figure 5.11).

5.7.8.3 Survival

Spearmans correlation demonstrated statistically significant corelations between EQ-5D, RMI, BADL, CFS, NHFS, HGS and low grip strength, ability to perform the TUG test and TUG time with 30-day mortality (Table 5.18).

Cox regression was undertaken with continuous variables to predict survival. All assumptions of the hazards proportional model were met. The overall model showed statistical significance (Table 5.19). Day 7 BADL and HGS demonstrated statistical

significance with a greater BADL score at day 7 and greater HGS predicting an improved survival.

Kaplan-Meier survival analysis demonstrates in Figure 5.12 as the CFS increases the survival decreases (log rank p=0.004). Observing the confidence intervals there is overlap between CFS 4 and 5 and CFS 6 and 7. Initially there is a severe decrease in survival in those with CFS 7 in the first 30 days. In this cohort although the NHFS also shows as the score increases the survival decreases this was not significant (log rank p=0.08) Figure 5.13

Patients who are unable to complete the TUG test have a lower survival compared to those who can complete the test Figure 5.14 Patients who have a low grip strength have a lower survival than those who have a normal grip strength Figure 5.15

In Figure 5.16 patients were placed into four groups normal grip strength and able to complete TUG test, normal grip strength and unable to complete TUG test, low grip strength and able to complete TUG test, and low grip strength and unable to complete TUG test. Patients who had a low grip strength and unable to complete the TUG test had the poorest survival. The remaining groups demonstrated a significant overlap of their confidence intervals suggesting no significant difference in survival.

ROC analysis

ROC analysis in Figure 5.17 shows the NHFS and CFS as a prediction tool for 30-day mortality, these are shown to be a good predictor based on the (AUC 0.687, [95%C.I. 0.524, 0.850]) p=0.024, (AUC 0.759, [95%C.I. 0.632, 0.885]) respectively. But the NHFS as previously stated was shown not to demonstrate a statistically significant difference in survival in this cohort. Figure 5.18 shows the ability to perform a TUG test, HGS and day 7 BADL to predict 30-day mortality. The maximum value for Youden's J statistic shows a cut off value of 32.50 for the BADL score would provide a 75.5% specificity and 100% sensitivity. The maximum value for Youden's J statistic shows a cut off value of 10.5kg for hand grip strength would provide a 83% specificity and 85.7% sensitivity.

5.7.9 Tables of results

Table 5.5 Correlations between Clinical frailty scale (CFS) Euro-Qol health-related quality of life (EQ-5D), subjective measures of frailty Groningen frailty index (GFI), and subjective and objective measures of muscle function Rivermead mobility index (RMI), Barthels activity of daily living (BADL), hand grip strength, timed up and go test, mid-thigh Vastus Lateralis muscle thickness). Abbreviations n=number; SD=standard deviation; SE=standard error. VLT-F=Vastus lateralis thickness fractured limb; VLT-NF= Vastus lateralis thickness non-fractured limb; TUG=timed up and go test; HGS= Hand grip strength; EWGSOP2= European Working Groups for Sarcopenia 2. *Spearmans correlation, **Pearsons correlation.

	EQ5D-Index		EQ5D-Health		GFI			RMI			BADL		
	Day 7	Day 90	Day 7	Day 90	Pre-Injury	Day 7	Day 90	Pre-Injury	Day 7	Day 90	Pre-Injury	Day 7	Day 90
CFS* (n)	(132)	(74)	(128)	(74)	(139)	(133)	(76)	(139)	(133)	(76)	(139)	(133)	(77)
Correlation coefficient	-.334	-.378	-.445	-.407	.435	.201	.436	-.641	-.433	-.448	-.711	-.575	-.428
Significance (2-tailed)	<.001	<.001	<.001	<.001	<.001	.020	<.001	<.001	<.001	<.001	<.001	<.001	<.001

	EQ5D-Index		EQ5D-Health		GFI			RMI			BADL		
	Day 7	Day 90	Day 7	Day 90	Pre-Injury	Day 7	Day 90	Pre-Injury	Day 7	Day 90	Pre-Injury	Day 7	Day 90
Low Grip Strength EWGSOP2*(n)	(130)	(74)	(126)	(74)	(133)	(131)	(76)	(133)	(131)	(76)	(133)	(131)	(77)
Correlation coefficient	-.287	-.312	-.424	-.367	.200	.151	.314	-.489	-.352	-.374	-.565	-.482	-.400
Significance (2-tailed)	<.001	.007	<.001	.001	.021	.086	.006	<.001	<.001	<.001	<.001	<.001	<.001
Ability to do TUG*(n)	(113)	(60)	(109)	(60)	(117)	(114)	(62)	(117)	(114)	(62)	(117)	(114)	(63)
Correlation coefficient	.416	.328	.351	.358	-.112	-.155	-.144	.357	.434	.416	.467	.599	.453
Significance (2-tailed)	<.001	.010	<.001	.005	.228	.100	.263	<.001	<.001	<.001	<.001	<.001	<.001

	EQ5D-Index		EQ5D-Health		GFI			RMI			BADL		
	Day 7	Day 90	Day 7	Day 90	Pre-Injury	Day 7	Day 90	Pre-Injury	Day 7	Day 90	Pre-Injury	Day 7	Day 90
HGS**(n)	(130)	(74)	(126)	(74)	(133)	(131)	(76)	(133)	(131)	(76)	(133)	(131)	(77)
Correlation coefficient	.323	.274	.285	.292	-.320	-.167	-.279	.523	.448	.383	.608	.571	.411
Significance (2-tailed)	<.001	.018	.001	.012	<.001	.056	.015	<.001	<.001	<.001	<.001	<.001	<.001
TUG time **(n)	(113)	(60)	(109)	(60)	(117)	(114)	(62)	(117)	(114)	(62)	(117)	(114)	(63)
Correlation coefficient	-.378	-.370	-.320	-.357	.152	.152	.170	-.343	-.436	-.424	-.444	-.595	-.503
Significance (2-tailed)	<.001	.004	<.001	.005	.102	.106	.186	<.001	<.001	<.001	<.001	<.001	<.001
VLT-F **(n)	(130)	(73)	(126)	(73)	(134)	(131)	(75)	(134)	(131)	(75)	(134)	(131)	(76)
Correlation coefficient	-.002	.020	.036	.229	-.210	.005	-.212	.139	.114	.119	.221	.202	.224

	EQ5D-Index		EQ5D-Health		GFI			RMI			BADL		
	Day 7	Day 90	Day 7	Day 90	Pre-Injury	Day 7	Day 90	Pre-Injury	Day 7	Day 90	Pre-Injury	Day 7	Day 90
Significance (2-tailed)	.981	.865	.690	.051	.015	.951	.068	.108	.193	.308	.010	.021	.052
VLT-NF **(n)	(132)	(74)	(128)	(74)	(136)	(133)	(76)	(136)	(133)	(76)	(136)	(133)	(77)
Correlation coefficient	.027	.204	.218	.358	-.188	-.021	-.327	.175	.197	.335	.220	.179	.299
Significance (2-tailed)	.757	.081	.014	.002	.028	.807	.004	.042	.023	.003	.010	.039	.008

Table 5.6 Mean Euro-Qol health-related quality of life (EQ-5D), subjective measures of frailty Groningen frailty index (GFI), and subjective and objective measures of muscle function Rivermead mobility index (RMI), Barthels activity of daily living (BADL), hand grip strength, timed up and go test, mid-thigh Vastus Lateralis muscle thickness). In each clinical frailty scale group. Abbreviations n=number; SD=standard deviation; SE=standard error. VLT-F=Vastus lateralis thickness fractured limb; VLT-NF= Vastus lateralis thickness non-fractured limb; TUG=timed up and go test; HGS= Hand grip strength. Between group statistical analysis was undertaken with ANOVA, where homogeneity of variance was met post-hoc testing was completed with Tukey HSD*. If homogeneity of variance was not met a Welch test was undertaken followed by Games-Howell** post hoc analysis demonstrated in Table 5.7.

	CFS					
	4	5	6	7	P value	F statistic
EQ5D-Index Day 7	0.391	0.210	0.182	0.134	.002*	F stat =5.085
EQ5D-Index Day 90	0.669	0.505	0.467	0.151	<.001**	F_{Welch} =7.891
EQ5D-Health Day 7	64.54	52.42	47.43	36.30	<.001*	F stat =9.452
EQ5D-Health Day 90	69.88	61.98	53.82	43.50	.003*	F stat =5.228
GFI Pre-Injury	5.69	7.16	7.90	9.31	<.001*	Fstat = 12.465

	CFS					
	4	5	6	7	P value	F statistic
Day 7	7.19	7.77	8.59	8.39	0.039*	Fstat = 2.872
Day 90	5.42	7.55	7.33	10.50	<.001*	Fstat = 8.771
RMI						
Pre-Injury	12.32 (1.621)	11.53 (1.984)	8.77 (3.445)	6.83 (3.413)	<.001**	F_{Welch} =32.923
Day 7	4.51 (2.321)	3.06 (2.016)	2.89 (2.246)	1.36 (1.615)	<.001**	F_{Welch} =12.550
Day 90	9.96 (3.638)	7.64 (3.430)	7.41 (4.836)	3.73 (2.724)	<.001*	Fstat =7.180
BADL						
Pre-Injury	97.56 (3.417)	95.16 (7.776)	84.62 (14.527)	57.93 (20.638)	<.001**	F_{Welch} =42.767
Day 7	64.86 (17.460)	51.94 (14.064)	46.62 (21.572)	22.68 (22.131)	<.001*	Fstat =26.620
Day 90	83.52 (20.466)	75.00 (21.712)	70.29 (33.656)	30.45 (20.181)	<.001**	F_{Welch} =17.663
Mean VLT-F (cm)	1.91 (0.46)	1.73 (0.45)	1.77 (0.57)	1.56 (0.54)	0.045*	Fstat =2.753
Mean VLT-NF (cm)	1.63 (0.53)	1.45 (0.36)	1.37 (0.42)	1.27 (0.48)	0.011**	F_{Welch} =3.106
TUG time (s)	344.97 (435.32)	479.76 (440.79)	661.53 (446.50)	904.03 (452.36)	<.001**	F_{Welch} =12.904

	CFS					
	4	5	6	7	P value	F statistic
Hand grip strength (Kg)	23.12 (8.18)	19.50 (5.91)	16.29 (7.59)	7.65 (8.16)	<.001*	Fstat =24.246

Table 5.7 This table demonstrates the post hoc testing and EQ5D-Index, EQ5D-Health, GFI, RMI, BADL, Mean VLT-F and VLT-NF, TUG time and HGS between CFS groups. Sig-Significant N.Sig-Not significant. Post hoc tests Tukey HSD*, Games-Howell**. It demonstrates which groups are statistically significantly different from each other and which ones are not.

	Sig	P value	95%CI	N.Sig	P value
EQ5D-Index Day 7*	4 and 6	0.013	0.033, 0.385	4 and 5	0.057
	4 and 7	0.004	0.064, 0.448	5 and 6	0.979
				5 and 7	0.758
				6 and 7	0.918
EQ5D-Index Day 90**	4 and 7	0.002	0.200, 0.835	4 and 5	0.130
	5 and 7	0.033	0.024, 0.683	4 and 6	0.270
				5 and 6	0.987
				6 and 7	0.139
EQ5D-Health Day 7*	4 and 6	0.003	4.53, 29.69	4 and 5	0.083
	4 and 7	<.001	13.87, 42.60	5 and 6	0.758
	5 and 7	0.028	1.22, 31.01	6 and 7	0.187
EQ5D-Health Day 90*	4 and 6	0.049	0.03, 32.09	4 and 5	0.516
	4 and 7	0.003	7.26, 45.51	5 and 6	0.579
				5 and 7	0.075
				6 and 7	0.549
GFI Pre-injury*	4 and 6	<.001	-3.67, -0.74	4 and 5	0.070
	4 and 7	<.001	-5.20, -2.03	5 and 6	0.596
	5 and 7	0.005	-3.81, -0.50	6 and 7	0.099
GFI Day 7*	4 and 6	0.003	-2.76, -0.05	4 and 5	0.707
				4 and 7	0.144
				5 and 6	0.438
				5 and 7	0.714
				6 and 7	0.984
GFI Day 90*	4 and 5	0.042	-4.19, -0.05	4 and 6	0.109

	Sig	P value	95%CI	N.Sig	P value
	4 and 7	<.001	-7.73, -2.42	5 and 6	0.995
	5 and 7	0.028	-5.68, -0.23		
	6 and 7	0.021	-5.98, -0.35		
RMI Pre-injury**	4 and 5	0.041	0.03, 2.34	6 and 7	0.107
	4 and 6	<.001	2.33, 5.56		
	4 and 7	<.001	4.05, 7.73		
	5 and 6	<.001	1.04, 4.49		
	5 and 7	<.001	2.77, 6.64		
RMI Day 7**	4 and 6	0.028	0.13, 3.11	4 and 5	0.058
	4 and 7	<.001	1.76, 4.55	5 and 6	0.987
	5 and 7	0.004	0.45, 2.96		
	6 and 7	0.011	0.27, 2.80		
RMI Day 90*	4 and 7	<.001	2.66, 9.81	4 and 5	0.155
	5 and 7	0.032	0.24, 7.58	4 and 6	0.143
				5 and 6	0.998
				6 and 7	0.065
BADL Pre-injury**	4 and 6	<.001	6.56, 19.34	4 and 5	0.375
	4 and 7	<.001	29.09, 50.18		
	5 and 6	0.001	3.40, 17.68		
	5 and 7	<.001	26.25, 48.20		
	6 and 7	<.001	14.75, 38.62		
BADL Day 7*	4 and 5	0.031	0.85, 25.01	5 and 6	0.663
	4 and 6	<.001	6.71, 29.78		
	4 and 7	<.001	29.76, 54.62		
	5 and 7	<.001	16.32, 42.19		
	6 and 7	<.001	11.51, 36.37		
BADL Day 90**	4 and 7	<.001	32.67, 73.46	4 and 5	0.505
	5 and 7	<.001	23.28, 65.82	4 and 6	0.477
	6 and 7	<.001	11.90, 67.78	5 and 6	0.958

	Sig	P value	95%CI	N.Sig	P value
Mean VLT-F	4 and 7	0.026	0.03,0.69	4 and 5	0.444
				4 and 6	0.613
				5 and 6	0.987
				5 and 7	0.565
				6 and 7	0.330
Mean VLT-NF	4 and 7	0.027	0.03, 0.69	4 and 5	0.381
				4 and 6	0.091
				5 and 6	0.794
				5 and 7	0.354
				6 and 7	0.823
TUG time (s)	4 and 6	0.034	-615.02, -18.10	4 and 5	0.672
	4 and 7	<.001	-823.58, -294.55	5 and 6	0.386
	5 and 7	<.001	-691.24, -157.29	6 and 7	0.052
Hand grip strength	4 and 6	<.001	2.24, 11.42	4 and 5	0.204
(Kg)	4 and 7	<.001	10.61, 20.34	5 and 6	0.309
	5 and 7	<.001	6.78, 16.92		
	6 and 7	<.001	3.75, 13.54		

Table 5.8 Mean difference in EQ5D-Index and Health from day 7 to day 90 within each frailty group. Statistical analysis with paired t-test.

	Index				Health			
CFS	Mean Δ (±SD)	t value	df	P value	Mean Δ (±SD)	t value	df	P value
4	0.298 (0.251)	5.936	24	<.001	3.92 (18.55)	1.079	25	.291
5	0.332 (0.349)	4.367	20	<.001	12.45 (32.31)	1.766	20	.093
6	0.310 (0.437)	2.921	16	.010	3.82 (34.69)	0.454	16	.656
7	-0.009 (0.361)	-0.087	10	.993	0.625 (18.60)	0.095	7	.927

Table 5.9 Mean difference in GFI, RMI and BADL within each frailty group comparing day 7 with pre-injury, day 90 with pre-injury, and day 90 with day 7. Statistical analysis with paired t test.

CFS		GFI Mean Δ (±SD)	t value	df	P value	RMI Mean Δ (±SD)	t value	df	P value	BADL Mean Δ (±SD)	t value	df	P value
4	Day 7 & Pre-injury	1.514 (2.835)	3.248	36	0.003	-8.243 (2.408)	-20.820	36	<.001	-32.703 (17.779)	-11.188	36	<.001
	Day 90 & Pre-injury	-0.423 (2.352)	-0.917	25	0.368	-2.769 (3.179)	-4.442	25	<.001	-13.704 (21.058)	-3.381	26	0.002
	Day 90 & Day 7	-1.808 (2.333)	-3.951	25	<.001	5.346 (2.770)	9.840	25	<.001	18.704 (18.584)	5.230	26	<.001
5	Day 7 & Pre-injury	0.516 (2.619)	1.097	30	0.281	-8.355 (2.811)	-16.547	30	<.001	-43.065 (15.528)	-15.441	30	<.001
	Day 90 & Pre-injury	0.409 (2.557)	0.750	21	0.461	-3.636 (3.125)	-5.458	21	<.001	-19.545 (21.208)	-4.323	21	<.001
	Day 90 & Day 7	-0.182 (2.905)	-0.294	21	0.772	4.773 (3.323)	6.737	21	<.001	23.636 (20.539)	5.398	21	<.001

CFS		GFI Mean Δ (±SD)	t value	df	P value	RMI Mean Δ (±SD)	t value	df	P value	BADL Mean Δ (±SD)	t value	df	P value
6	Day 7 & Pre-injury	0.757 (3.022)	1.523	36	0.136	-6.054 (2.999)	-12.277	36	<.001	-38.378 (19.078)	-12.237	36	<.001
	Day 90 & Pre-injury	-0.722 (2.608)	-1.175	17	0.256	-1.471 (3.281)	-1.848	16	0.083	-13.824 (24.719)	-2.306	16	0.035
	Day 90 & Day 7	-2.222 (3.001)	-3.142	14	0.006	4.353 (4.663)	3.849	16	0.001	18.824 (31.201)	2.487	16	0.024
7	Day 7 & Pre-injury	-0.750 (3.296)	-1.204	27	0.239	-5.500 (3.575)	-8.142	27	<.001	-35.000 (23.609)	-7.844	27	<.001
	Day 90 & Pre-injury	0.700 (2.003)	1.105	9	0.298	-4.727 (3.901)	-4.019	10	0.002	-35.000 (19.621)	-5.916	10	<.001
	Day 90 & Day 7	2.000 (2.404)	2.631	9	0.027	2.273 (3.663)	2.058	10	0.067	1.364 (25.990)	0.174	10	0.865

Table 5.10 Correlation table of frailty and objective measures of muscle function. Note. *Spearmans Correlation, ** Pearsons Correlation.

	VLT-F	VLT-NF	HGS	Low grip strength (EWGSOP2)	Ability to do TUG	TUG time
CFS (n)	134	136	133	133	117	117
Correlation coefficient	-.249*	-.252*	-.576*	.512*	-.444*	.459*
Significance (2-tailed)	.004	.003	<.001	<.001	<.001	<.001
VLT-F(n)		134	131	131	115	115
Correlation coefficient		.571**	.282**	-.237*	.029*	-.019**
Significance (2-tailed)		<.001	.001	.006	.756	.841
VLT-NF(n)			133	133	117	117
Correlation coefficient			.288**	-.317*	.022*	-.044**
Significance (2-tailed)			<.001	<.001	.816	.640
HGS				133	115	115
Correlation coefficient				-.639*	.319*	-.331**
Significance (2-tailed)				<.001	<.001	<.001

	VLT-F	VLT-NF	HGS	Low grip strength (EWGSOP2)	Ability to do TUG	TUG time
Low grip strength (EWGSOP2)					115	115
Correlation coefficient					-.418*	.373*
Significance (2-tailed)					<.001	<.001
Ability to complete TUG						115
Correlation coefficient						-.948*
Significance (2-tailed)						<.001

Table 5.11 Ability to complete TUG test and frailty score cross tabulation.

		Able to complete TUG		
		No	Yes	Total
CFS	4	8	19	27
	5	11	16	27
	6	22	13	35
	7	25	3	28
Total		66	51	117

Table 5.12 Post operative complications following hip fracture surgery

Complications	n
Pulmonary embolus	2
Pneumonia	23
Urinary tract infection	13
Cerebrovascular accident	3
Myocardial infarction/ Acute coronary syndrome	1
Deep vein thrombosis	0
Acute delerium	23
Acute kidney injury	35
Post operative anaemia	84
Blood transfusion required	15
Dislocation	0
Failure of fixation	3
Wound problems	1
Surgical site infection	2
Reoperation in 120 days	3
Pressure ulcer	1
C.difficle infection	0
Inpatient mortality	12

Table 5.13 Spearmans* correlation table for the development of 1 or more post operative complication with subjective and objective measures of performance. Mean EQ5D-Index, EQ5D-Health, GFI, RMI, BADL, Vastus lateralis thickness and ability to complete TUG test, TUG time, HGS low grip strength. Grip strength cut offs according to EWGSOP2 criteria.

	Day	Development of 1 or more complication* (n)	Correlation coefficient	Significance (2-tailed)
EQ5D-Index	7	(132)	-.146	.094
	90	(74)	.083	.480
EQ5D-Health	7	(128)	-.136	.126
	90	(74)	.070	.553
GFI	Pre-Injury	(139)	-.031	.719
	7	(133)	.116	.183
	90	(76)	-.038	.745
RMI	Pre-Injury	(139)	-.055	.523
	7	(133)	-.061	.482
	90	(76)	-.038	.742
BADL	Pre-Injury	(139)	-.022	.801
	7	(133)	-.138	.113
	90	(77)	-.033	.774
CFS		139	.093	.274
HGS		133	-.116	.182
Low grip strength EWGSOP2		133	.125	.152
Able to complete TUG		117	-.080	.390
TUG time		117	.093	.318
VLT-F		134	-.021	.808
VLT-NF		136	-.143	.098

Table 5.14 Crosstabulation pre-injury residence and discharge destination

		Discharge destination					
		Home	Rehab	Residential home	Nursing home	Deceased whilst inpatient	Total
Pre-injury residence	Home	71	30	5	0	10	116
	Residential Home	0	0	11	0	2	13
	Nursing Home	0	0	0	9	1	10
	Total	71	30	16	9	13	139

Table 5.15 *Spearmans corelation table demonstrating discharge destination with subjective and objective measures of muscle function and frailty score.

	Discharge home (n)*	Correlation coefficient	Significance (2-tailed)
EQ5D Index- day 7	(110)	.401	<.001
EQ5D Health- day 7	(110)	.363	<.001
GFI Pre-Injury	(116)	-.153	.100
GFI day 7	(110)	-.171	.073
RMI Pre-Injury	(116)	.211	.023
RMI day 7	(110)	.444	<.001
BADL Pre-Injury	(116)	.176	.059
BADL day 7	(110)	.532	<.001
CFS	(111)	-.378	<.001
HGS	(111)	.117	.222
Low grip strength (EWGSOP2)	(111)	-.232	.014
Ability to do TUG	(95)	.582	<.001
TUG time	(95)	-.576	<.001
VLT-F	(112)	.071	.457
VLT-NF	(113)	.100	.290

Table 5.16 Logistic regression classification tables to ascertain the effects of CFS, grip strength according to EWGSOP2 guidelines and ability to perform timed up and go test on the likelihood that patients admitted with a hip fracture would be discharged back to their own home. (For full tables see Appendix 9.1.2.10)

Variables in the Equation		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B)	
								Lower	Upper
Step 1[a]	CFS			3.858	3	.277			
	CFS(1)	-1.140	.748	2.324	1	.127	.320	.074	1.385
	CFS(2)	-1.379	.746	3.419	1	.064	.252	.058	1.086
	CFS(3)	-1.230	.911	1.824	1	.177	.292	.049	1.742
	Low HGS EWGSOP2(1)	-.047	.568	.007	1	.934	.954	.313	2.906
	Able to complete TUG(1)	2.580	.566	20.767	1	<.001	13.195	4.350	40.023
	Constant	.104	.656	.025	1	.875	1.109		
a. Variable(s) entered on step 1: CFS, Low HGS EWGSOP2, Able to complete TUG.									

Table 5.17 Receiver operator characteristic table and Youden's J Statistic for the ability to perform TUG test and Day 7 BADL for the ability to predict discharge home following admission from home. (For full tables see Appendix 9.1.2.10)

Area Under the ROC Curve					
Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
D7 BADL	.807	.046	.000	.717	.896
Able to complete TUG	.788	.050	.000	.690	.885
a. Under the nonparametric assumption					
b. Null hypothesis: true area = 0.5					

Table 5.18 *Spearmans corelation table demonstrating 30-day mortality with subjective and objective measures of muscle function, frailty score and Nottingham Hip Fracture Score.

	Mortality 30 day (n)*	Correlation coefficient	Significance (2-tailed)
EQ5D Index- day 7	(132)	-.272	.002
EQ5D Health- day 7	(128)	-.306	<.001
GFI Pre-Injury	(139)	.101	.239
GFI day 7	(133)	-.008	.927
RMI Pre-Injury	(139)	-.202	.017
RMI day 7	(133)	-.222	.010
BADL Pre-Injury	(139)	-.231	.006
BADL day 7	(133)	-.342	<.001
CFS	(139)	.250	.003
NHFS	(139)	.181	.033
HGS	(133)	-.338	<.001
Low grip strength (EWGSOP2)	(133)	.263	.002
Ability to do TUG	(117)	-.254	.006
TUG time	(117)	-.241	.009
VLT-F	(134)	-.126	.148
VLT-NF	(136)	-.045	.601

Table 5.19 Cox regression tables for continuous variables to predict survival. (For full tables see Appendix 9.1.2.10)

Variables in the Equation								
	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
D7EQ5D Index	-.157	.708	.049	1	.825	.855	.213	3.426
D7 EQ5D Health	.007	.008	.606	1	.436	1.007	.990	1.023
Pre-injury RMI	-.103	.064	2.575	1	.109	.902	.796	1.023
D7 RMI	.071	.115	.381	1	.537	1.074	.857	1.346
Pre-injury BADL	.013	.013	1.098	1	.295	1.013	.988	1.039
D7 BADL	-.032	.014	4.829	1	.028	.969	.942	.997
Best HGS	-.063	.029	4.816	1	.028	.939	.888	.993
TUG time	.000	.000	.011	1	.916	1.000	.999	1.001

5.7.10 Figures of results

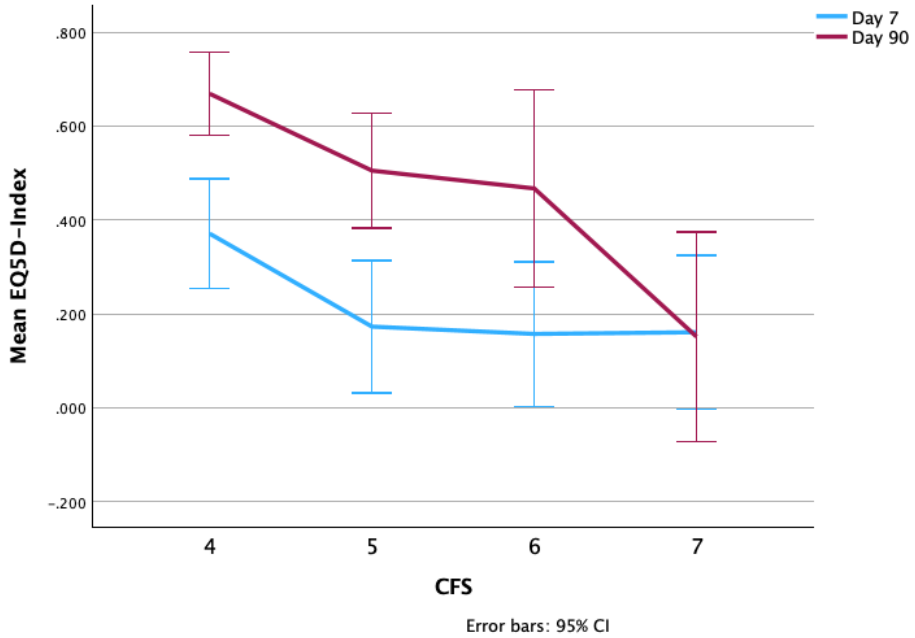


Figure 5.2 *Mean day 7 and day 90 EuroQol-5D -Index values within each frailty group*

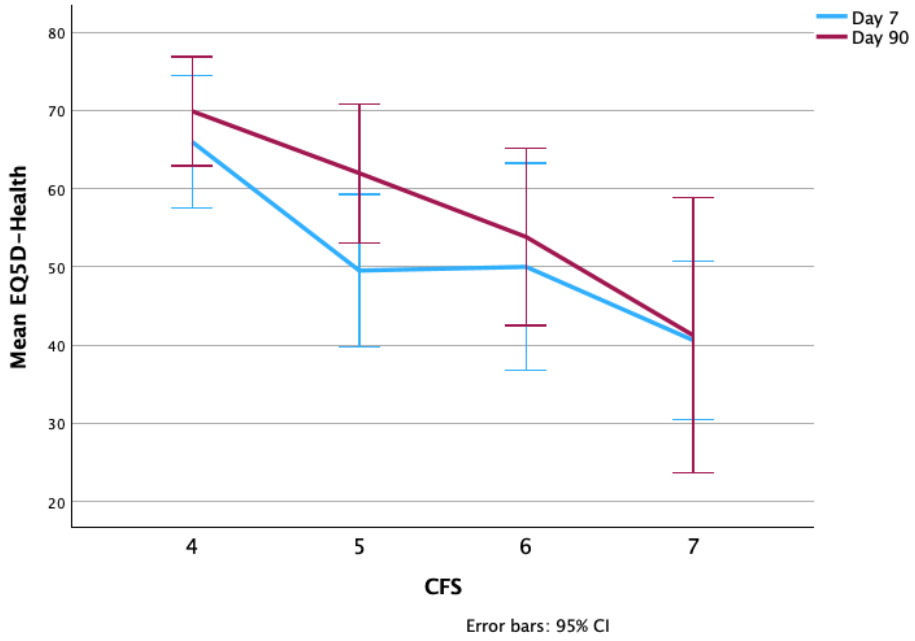


Figure 5.3 *Mean day 7 and day 90 EuroQol-5D -Health values within each frailty group*

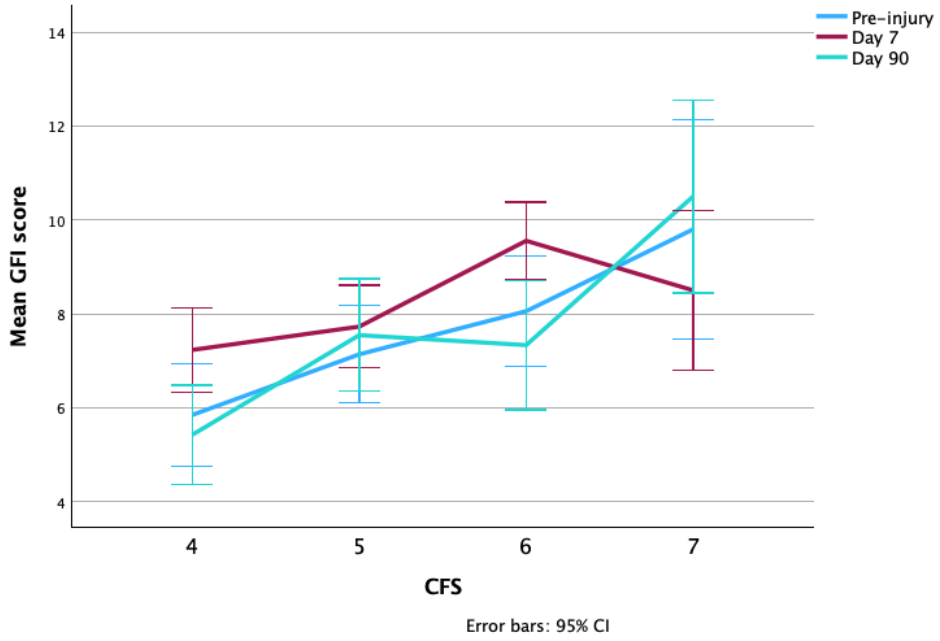


Figure 5.4 *Mean Groningen frailty index vs Clinical frailty scale score*

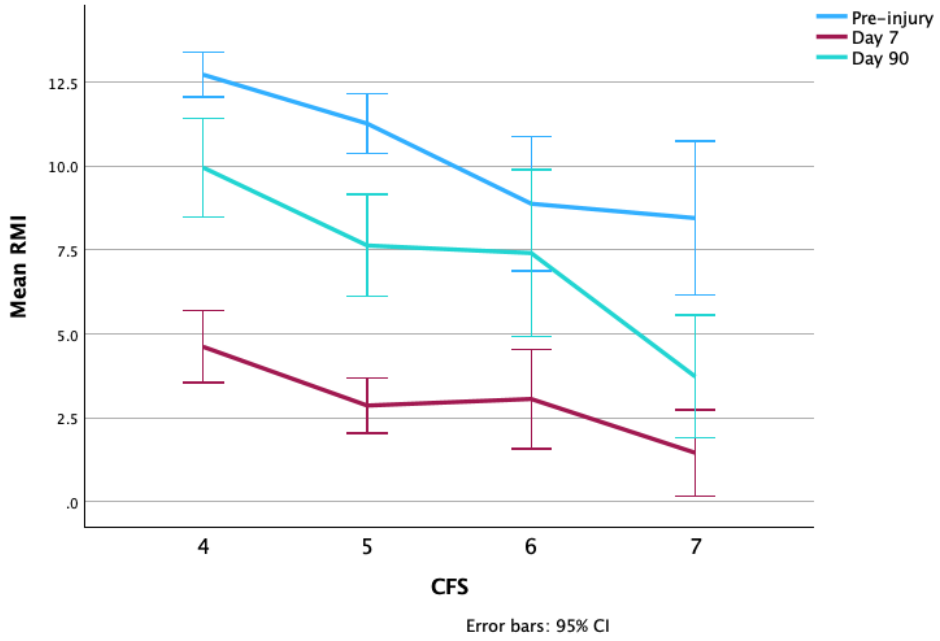


Figure 5.5 *Mean Rivermead mobility index (pre-injury, day 7 and day 90) and Clinical frailty score*

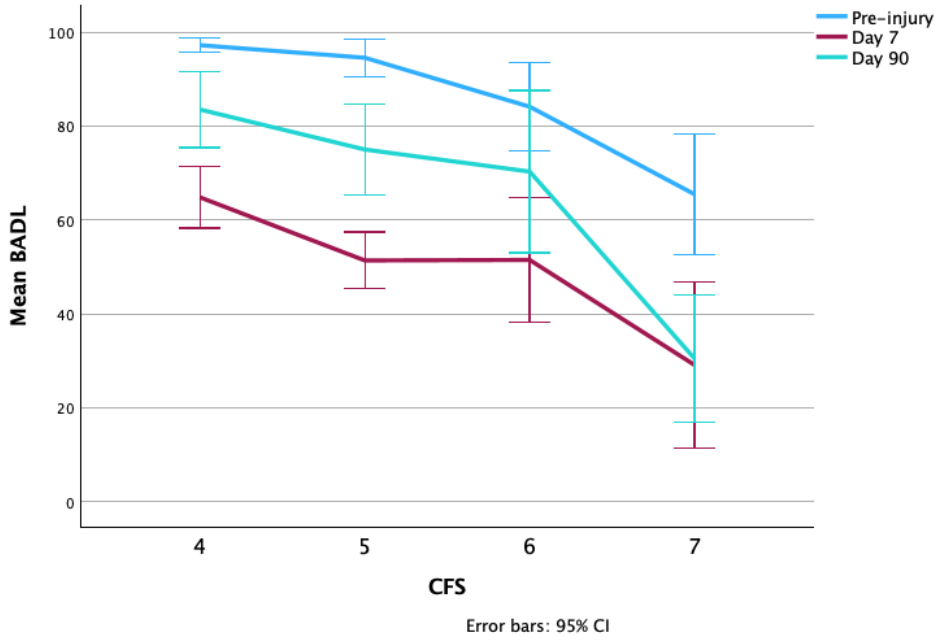


Figure 5.6 *Mean Barthels activity of daily living (pre-injury, day 7 and day 90) and clinical frailty scale score*

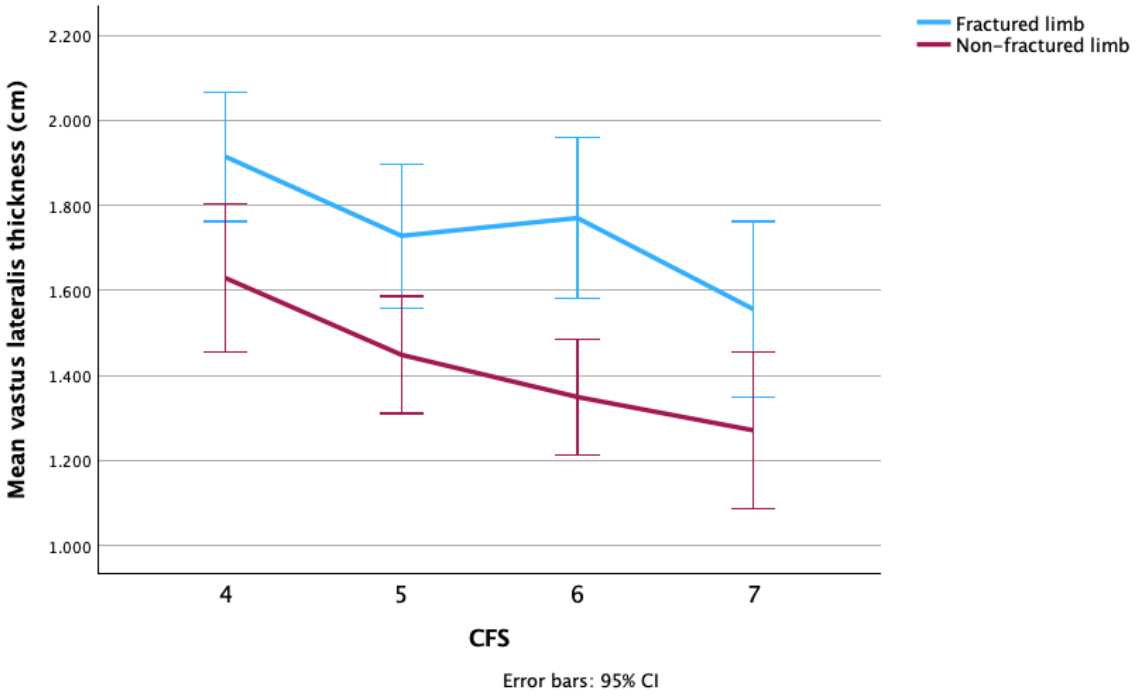


Figure 5.7 *Mean vastus lateralis thickness in both the fractured limb and non-fractured limb and CFS*

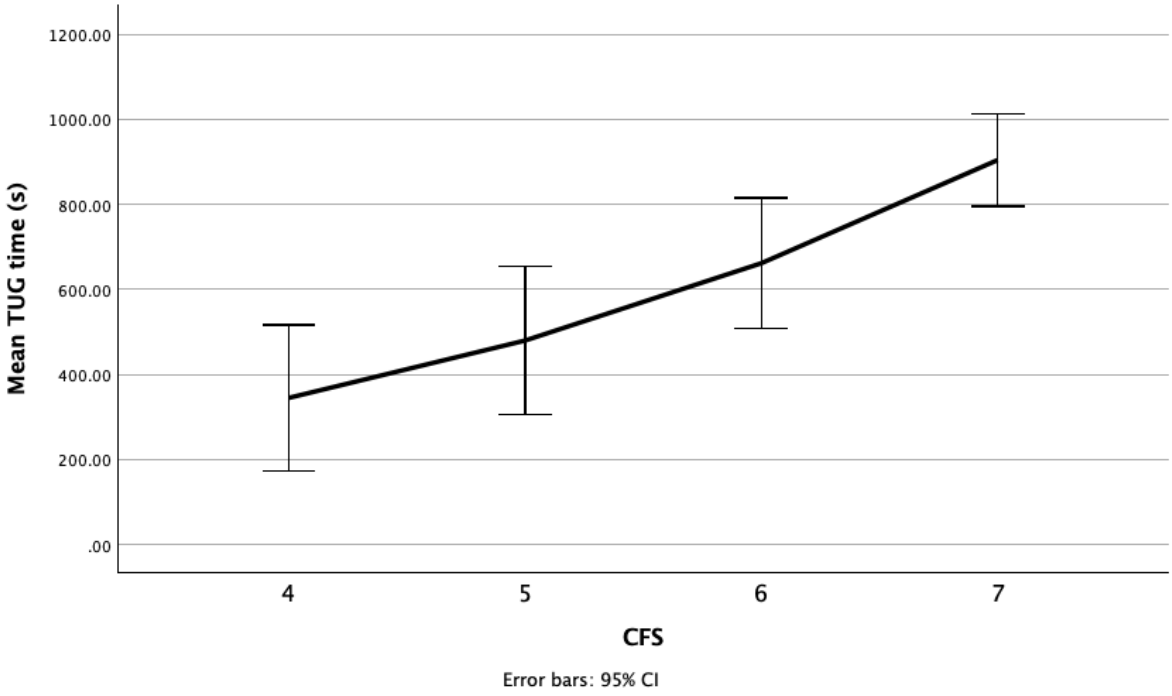


Figure 5.8 *CFS and TUG time*

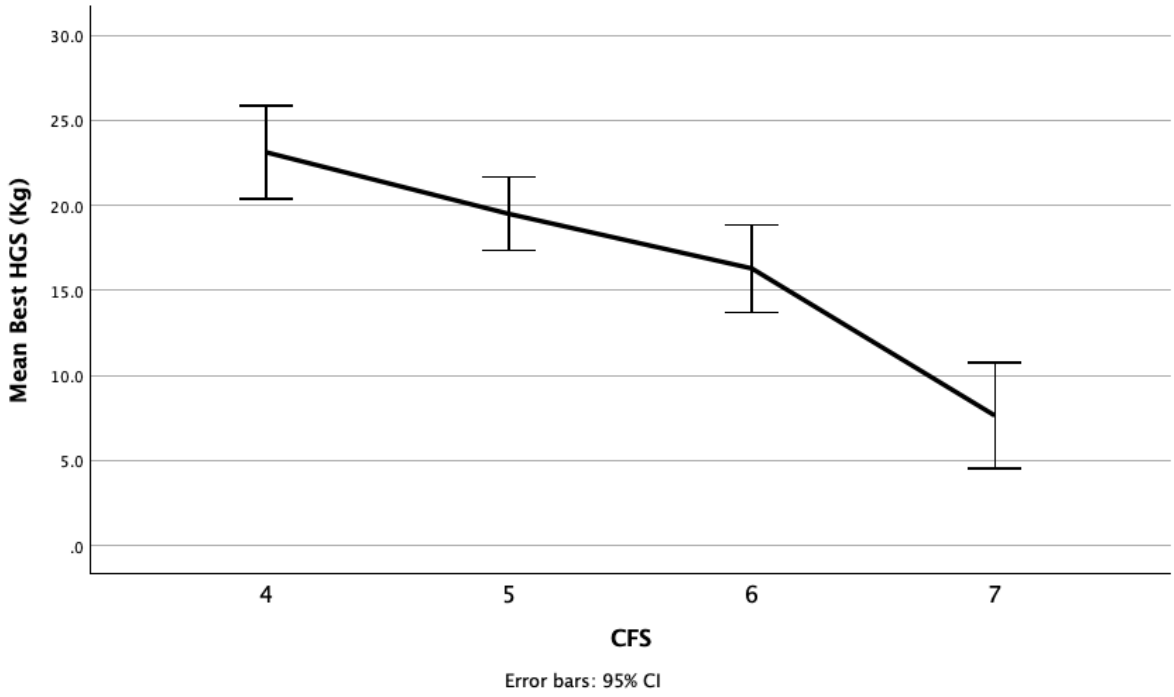


Figure 5.9 *Mean best hand grip strength and CFS*

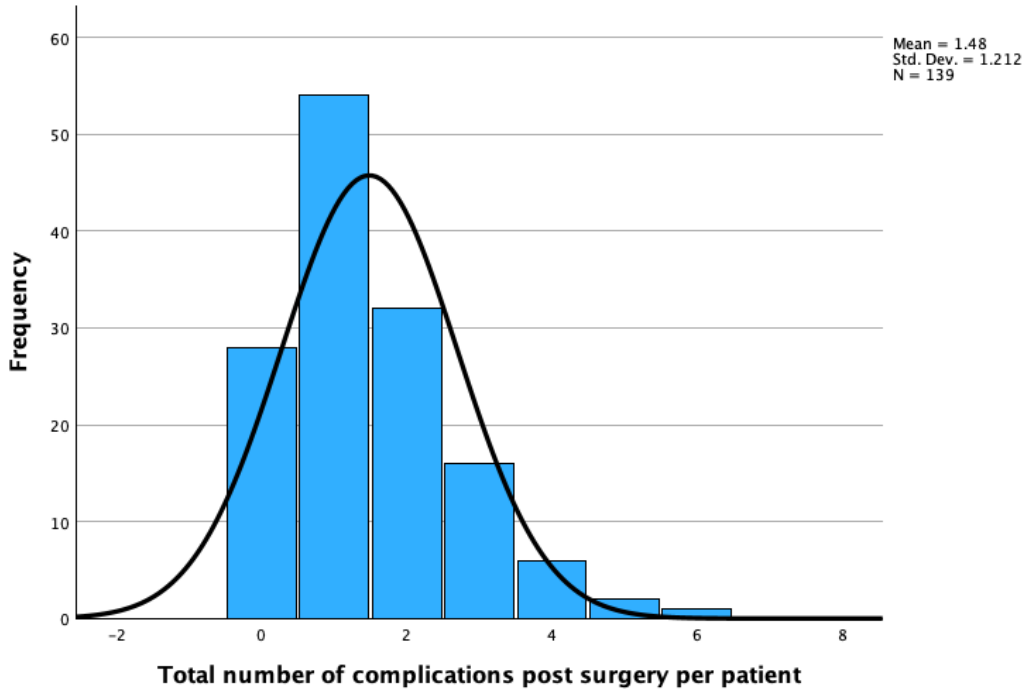


Figure 5.10. Histogram of f*requency of total number of complications post-surgery per patient*

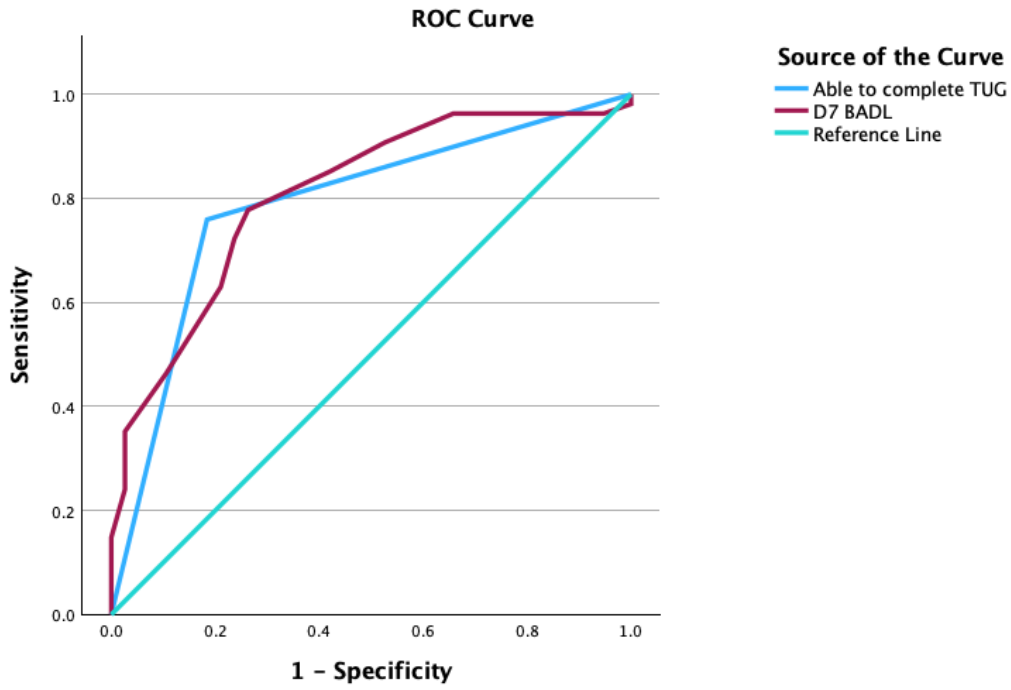


Figure 5.11 ROC curve ability to perform TUG and day 7 BADL score and discharge home

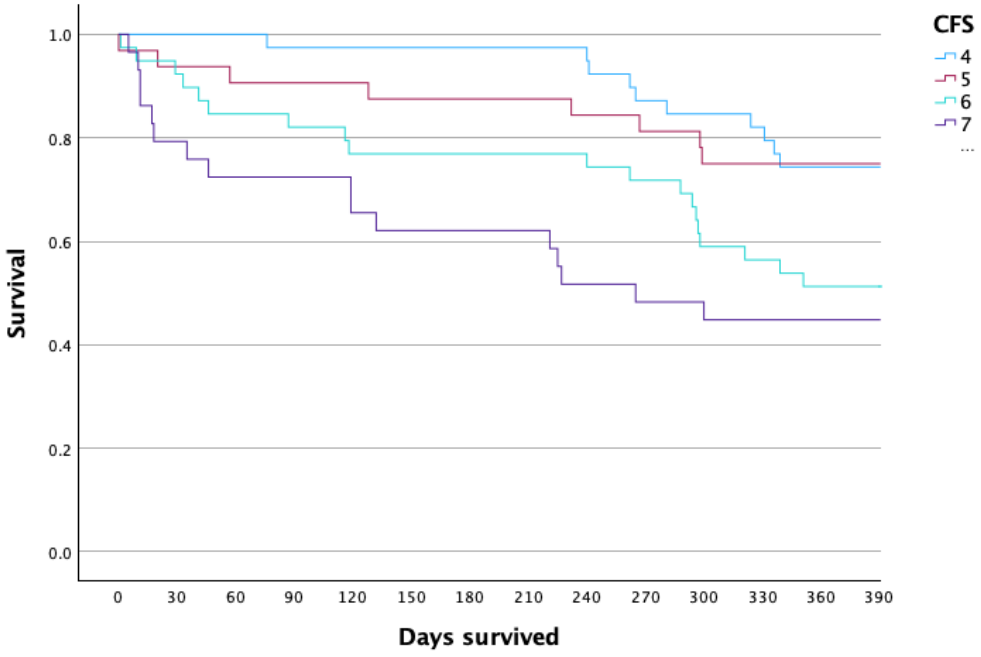


Figure 5.12 *Kaplan-Meier survival curve stratified for Clinical Frailty Score*

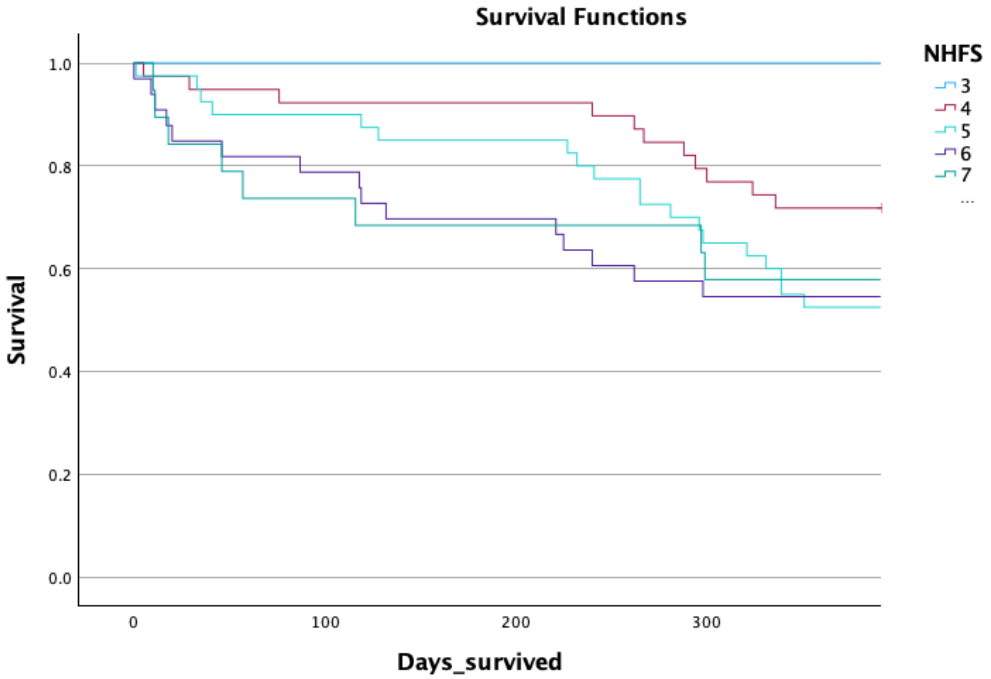


Figure 5.13 Kaplan-Meier Survival curve for the Nottingham hip fracture score

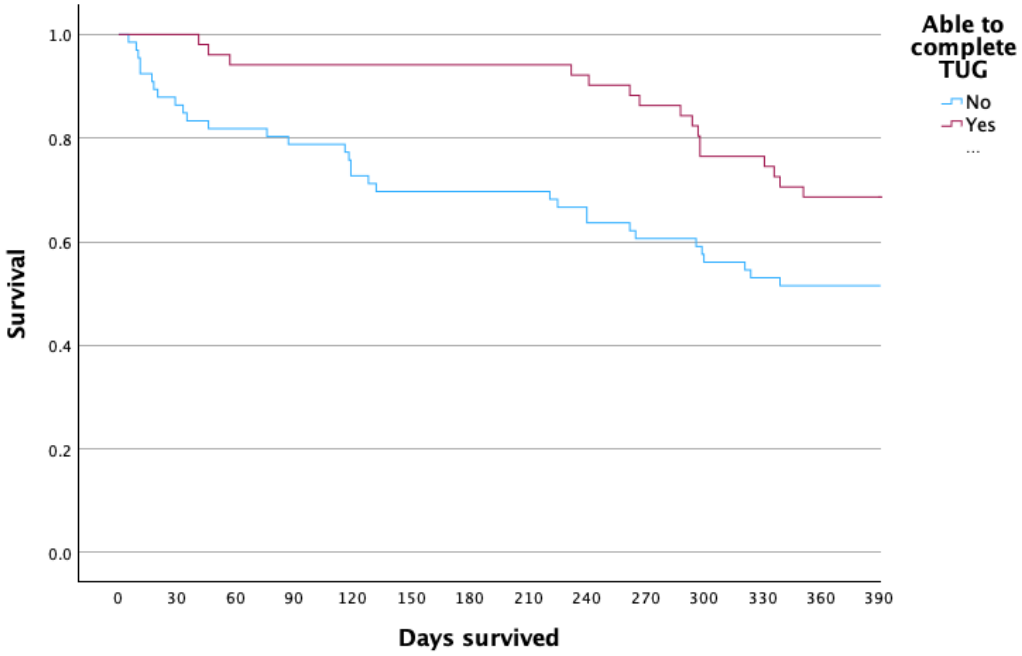


Figure 5.14. *Kaplan-Meier survival curve Ability to perform Timed up and go test*

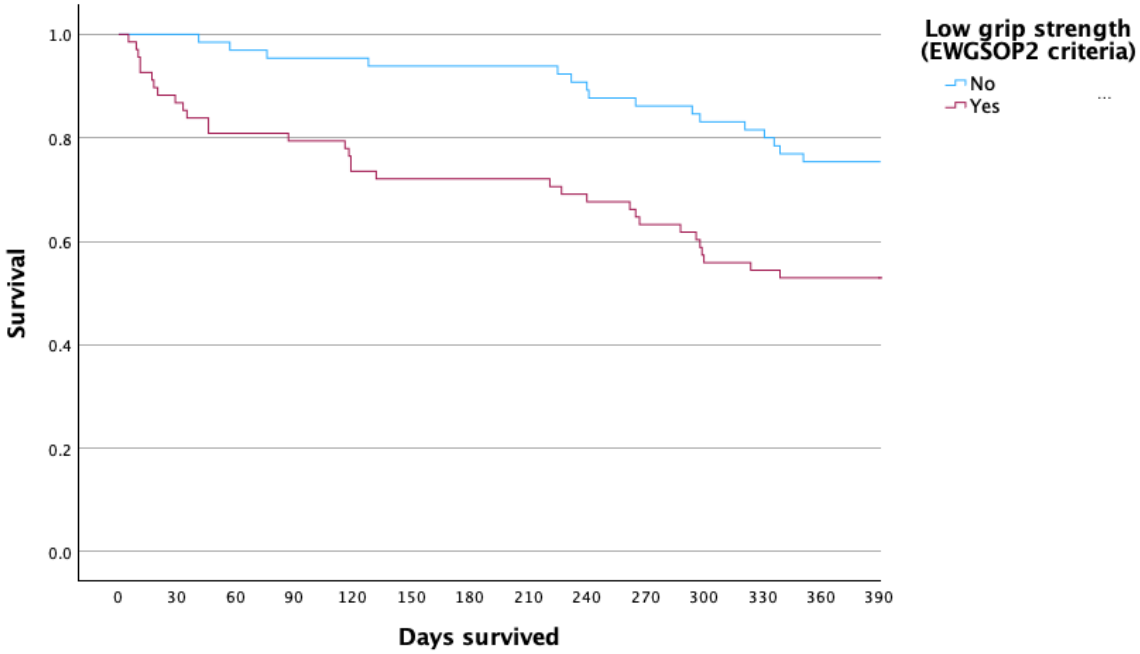


Figure 5.15. *Kaplan-Meier survival curve Low grip strength EWGSOP2 criteria*

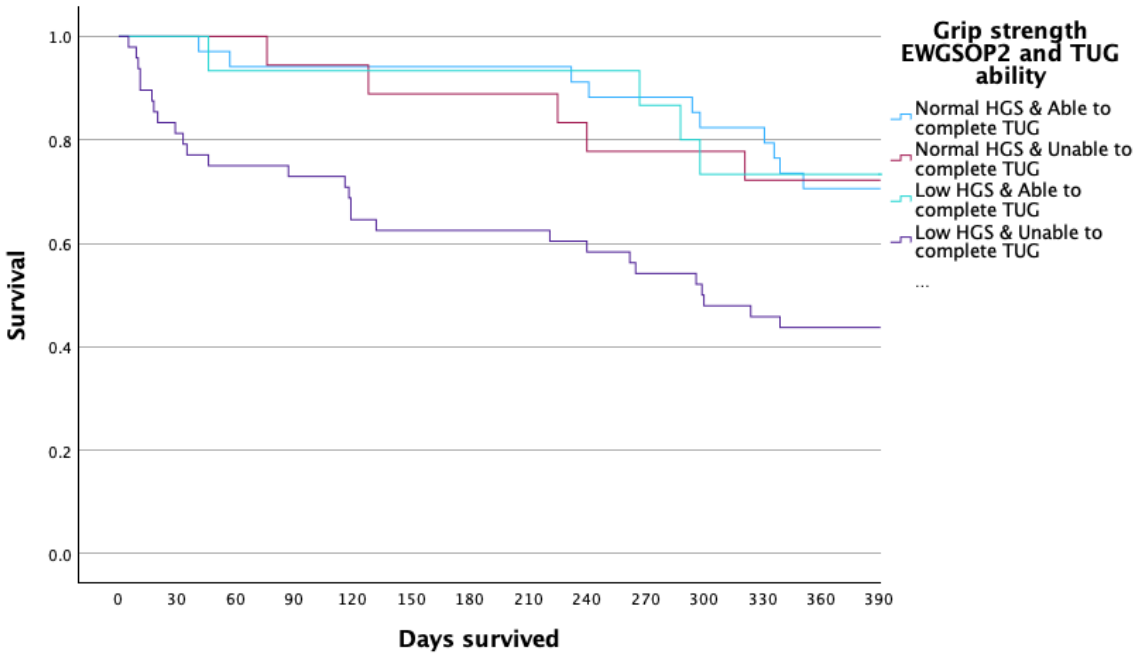


Figure 5.16 *Kaplan-Meier survival curve Low grip strength EWGSOP2 criteria and TUG test ability*

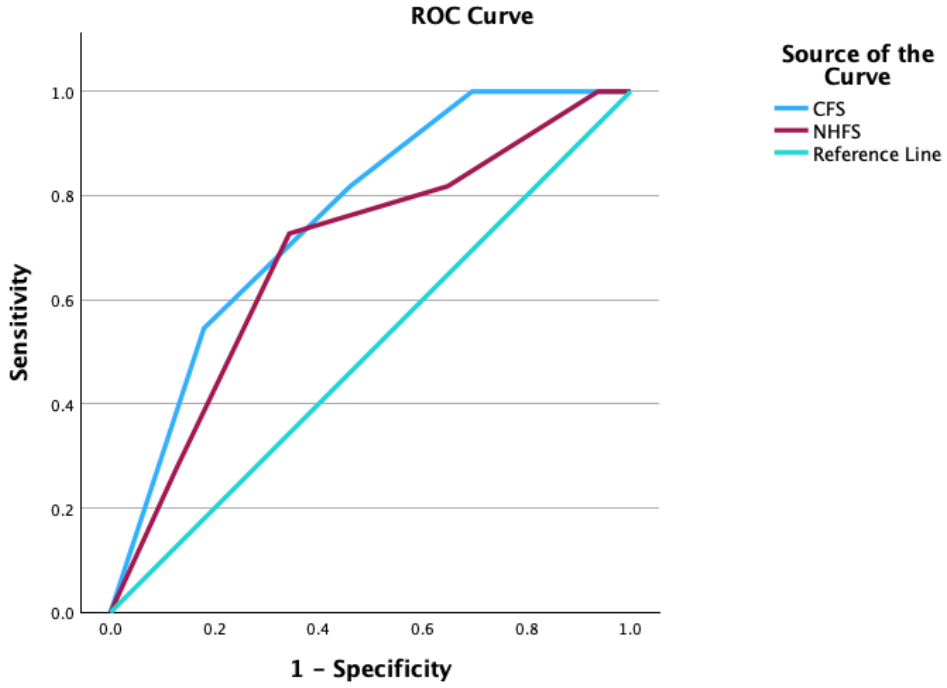


Figure 5.17. Receiver operator characteristic curve comparing clinical frailty scale score and the Nottingham Hip Fracture Score CFS for 30-day mortality

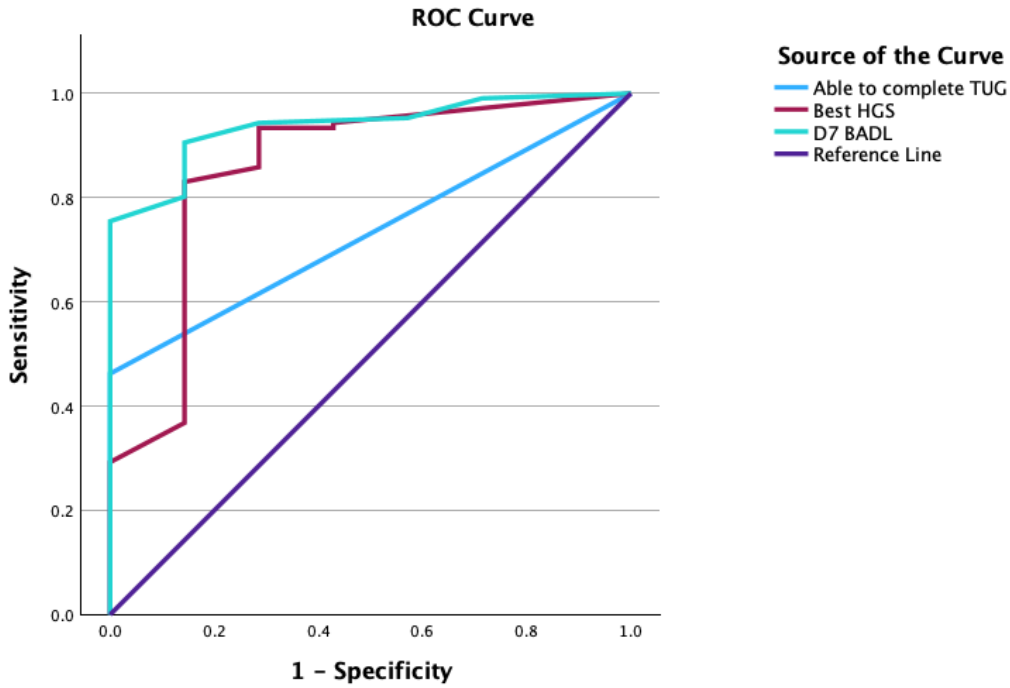


Figure 5.18. Receiver operator characteristic curve Ability to perform Timed up and go test, HGS and Day 7 Barthels activity of daily living for 30-day mortality.

5.8 Discussion

5.8.1 Clinical frailty and subjective measures of function

5.8.1.1 EQ5D

Hip fracture affects all five domains of the EQ5D; pain and discomfort, mobility, self-care, usual activities, and anxiety and depression. This observation is in line with expectations as hip fracture is a major health insult and EQ-5D is validated as a global health related quality of life measure. Over the course of recovery following a hip fracture the ability to complete these domains would be expected to improve. A value of 1 in the EQ-5D index indicates full health whereas a value lower than 0 represent a health state worse than being dead.

In this study overall the EQ-5D was better at day-90 follow up than at day-7. The day-7 EQ-5D was undertaken soon following surgery. In this time patients have begun the process of coming to terms with the injury, surgery, and immediate loss of independence but also the psychological aspect of trauma.(268) At this point there is uncertainty of what the future may hold with regards to their recovery and rehabilitation. This may explain poorer scores with regards to anxiety and depression and usual activities. These patients may also have had concurrent illnesses contributing to the fall such as delirium and infection along with pain which may have influenced or compounded to result in a lower score. (269-271) Many of these patients especially those who are cognitively intact may deem themselves to be ferociously independent. Despite the physical manifestation of frailty and reduced muscle reserve, they have been living alone and coping with their activities of daily living. Following a hip fracture this may be the first time their independence and level of frailty has come under scrutiny. Many of their activities may be limited in the initial phase of recovery due to recent surgery which would involve soft tissue dissection to fix or replace the fracture and pain. This may explain poor scores in pain and discomfort, mobility and self-care. The pain despite adequate analgesia is usually exacerbated by the active urgent physiotherapy needed to prevent immobility and sequalae of hip fracture morbidity. It has been shown to lead to a delay in mobilisation and decreased patient satisfaction.(90) However, this would have been lower than the

pain associated with an untreated hip fracture. With time the EQ-5D improved showing patients global health did recover specifically the index values, but the health (visual analogue scale) did not show an improvement. This visual element of the assessment in this cohort of patient may be difficult to understand, interpret, see, or in fact without original reference value from day-7, place into perspective a value for day-90 for both the patient and or consultee. (271) If the patient's frailty and cognition changed from pre-admission to day-90 the EQ-5D health may have been completed by a consultee or proxy rather than the patient themselves or vice versa. However, the EQ-5D has been shown in literature to be robust whether the patient or proxy completes the form, but it is unknown if the same proxy is required to complete the form for this to hold true.(83)

The observed improvements in EQ-5D during recovery after a hip fracture are not a new finding, however they are independent to other studies and can be used to characterise recovery trajectory and validate the scale further for use in hip fracture.

When analysing the EQ-5D with regards to the clinical frailty scores, patients with a lower frailty scale score had a better EQ-5D index both at day-7 and day-90 but this was a weak correlation. Post-hoc analysis demonstrated there are significant differences in health-related quality of life at between the two ends of the frailty spectrum particularly CFS4 and CFS7. This holds true for the EQ-5D index and the EQ-5D health visual analogue scale completed at day-7 and day-90. There was no statistically significant difference between patients in adjacent CFS groups. Patients with a CFS 4, 5 and 6 demonstrate some improvement in EQ-5D index from day 7 to day 90 with an increase in mean score 0.298, 0.332, and 0.310 respectively. However, patients with a CFS 7 show no change in quality of life from day 7 to day 90. This informs us, jumps or significant increases in frailty states of more than one CFS correlates with poorer EQ5D outcomes.

When stratified for CFS groups, changes in frailty levels showed no significant improvement in EQ-5D health from day 7 to day 90 reinforcing the visual analogue scale not to be a good discriminator in assessing global health assessment in this

cohort of patients. Data from this study aligns with existing literature supporting the use of EQ5D index as a measure of global health assessment but the visual analogue scale in the frail elderly appears to be problematic.(271) One of the limitations of this study is that the EQ5D was not completed on admission to ascertain pre-injury level of health state. However, this would have been difficult to accomplish as it would have led to recall bias. On admission to the emergency department at times it is difficult to obtain an adequate history from the patient due to cognitive impairment such as dementia, delirium or acute infection. For these patients this assessment may have proven difficult as caregivers may not have accompanied the patient to the emergency department and or next of kin not available until admission to the ward or at all. This was particularly evident especially during the Covid-19 pandemic to minimise transmission of the virus.

Another potential limitation inherent in the study methodology is the classification of frailty. Patients are admitted following an injury, and sometimes a 'long lie' and were assessed by the clinical team at the time of admission. It may be difficult for the orthopaedic team to undertake an assessment of frailty on admission in a short period of time and prior to resuscitation which can take several days.(102) As the patients were recruited from a tertiary Major trauma centre the number of patients reviewed by the on call orthopaedic team is significantly greater than most units in the country. In this study these biases were minimised as the frailty scale score was completed by a member of the research team familiar with and has undergone training with the orthogeriatric clinicians in the Rockwood scoring system. Time was spent to review the clinical notes and discuss with the appropriate clinical teams and any caregivers before assigning a CFS score. The EQ5D is a snapshot at the time the questionnaire was completed whereas the CFS is a holistic assessment of the patient over the preceding two weeks. Correlation of the day-90 EQ-5D with the admission CFS may be an unfair comparison as these were completed over two different time points and may be subject to recall bias. It may be deemed more appropriate to compare it to a repeat CFS at day 90. However, this would be challenging to undertake in the NHS setting as hip fractures in this cohort are not routinely followed up based upon existing evidence based clinical guidelines.

Concurrent illness may also have had a contributing factor to the EQ5D assessment and poorer scores at day 7. Hip fracture may be such a significant injury and impact to a patient, that subtle differences in frailty states i.e. between adjacent frailty scores are not noticeable and only at the extreme ends of frailty are the impacts of a hip fracture appreciated and fully observed with regards to the selected outcome measures. Patients may also not view their frailty to have an impact on their perception of their health as it is 'normal' for them. Rather they see frailty as an underlying disability as opposed to the ability to bounce back following injury or infection. This is common even amongst clinicians and several tools have been formulated for the clinical setting to help clinicians with this when assessing for frailty.(42) People with disability still may view themselves as having a poor or good quality of life despite the disability.

5.8.1.2 GFI

The clinical frailty scale score is an objective measure which usually requires an in-depth assessment before the allocation of a score, one of the ways this study analyses frailty is using a subjective measure such as the GFI. Traditionally the GFI uses a cut off value of 4 to define frailty. Those scoring more than 4 are frail and those under not frail. There are no subcategories of frailty within this scoring system. But, since a score is allocated, this could be used as a measure. Observing the results in entirety the GFI showed a significant increase from admission to day-7 showing a worsening of frailty. However, at day-90 there was no overall significant change from pre-injury levels.

In this study the admission CFS was compared with the preinjury, day 7 and day 90 GFI and have shown a significant positive weak correlation between admission CFS and GFI at day 7 but moderately positive at pre-injury and day 90. Observing the post-hoc tests it appears a significant change in GFI must occur before there is an objective change in CFS level. This implies a significant subjective change (as measured by the GRI) also relates to an objective change (as measured by the CFS) in frailty. Patients with a CFS 4-6 return to their pre-injury level of subjective frailty but those with a CFS 7 have a worsening frailty subjectively. This may push them into the next CFS frailty

subgroup from an objective perspective, but this has not been measured as it falls outside the scope of this PhD. CFS was not completed on day 7 as correct administration of the scale should reflect the patient's for the preceding two weeks and therefore not appropriate or applicable to be completed at this time. The CFS was not repeated at day 90 as it is an objective measure of frailty which requires a comprehensive assessment. Lastly follow up at this stage is limited due to the natural mortality of hip fracture with 8.3% dying in the first month(6), non-completion of the form due to difficulties in reading the form cognitive impairment, institutions being too busy with the care of care home residents or forms which have been completed but not returned may have affected the results via response rate.

5.8.1.3 RMI

Patients in all frailty groups had a significant drop in RMI from preinjury to day 90 except those with CFS6 who returned to their baseline. Patients with severe frailty CFS 7 statistically did not improve from day 7 post-surgery. CFS 4-5 demonstrated capacity for improvement from day 7-90 and the lower the frailty score the greater the improvement of RMI score.

This highlights the significant and important impact of being severely frail on mobility following hip fracture surgery and fits with the current literature of poorer mobility outcomes in increasingly frail patients.(272) Although patients with a CFS 6 return to their baseline mobility, their initial starting mobility was lower than that for CFS 4 and 5. Having a lower pre-injury frailty appears to allow patients to have a greater improvement in mobility and the lower the frailty the more a patient can return closer to their original level of mobility, again validating the concept of frailty in these patients. In CFS 4 and 5 those patients may not have returned to their pre-injury state at 3 months possibly due to needing more time to achieve this higher goal. Another reason could be not having enough extensive rehabilitation in the early phases of recovery, literature has shown that improving day 1 mobilisation improves long term outcomes,(90) this associated with acute muscle decline in the first two weeks which may occur, may impeded their ability to achieve a return to a higher level of function.(273)

Patients with severe frailty CFS 7 do not improve with regards to mobility after the first seven days following surgery and show no significant difference at day 90 when compared with day 7. This could be due to firstly the higher one-month mortality rate following hip fracture in this subgroup reducing the number of patients for the sub-group analysis. In this subgroup of patients, the insult of a hip fracture and immobility in the first week following may be too great an injury to bounce back from. The surgery involved to treat the hip fracture especially during the approach to the hip may impact the functional reserve of the muscle. The muscles around the hip may not have the capacity to cope with this especially during a trans vastus muscle split approach as opposed to a sub-vastus muscle sparing for a DHS fixation of an extracapsular hip fracture or an anterolateral approach through the abductors of the hip v Watson Jones muscle sparing approach in a hemi arthroplasty. Using muscle sparing techniques during surgery may help the functional outcomes especially in patients with already low functional reserve. Recently it has been established that direct anterior approach has led to increasingly successful mobility outcomes and reduced length of stay in patients for elective hip replacements(274) but long term outcomes remain similar for this patient group,(275, 276) however this elective group of patients are not usually as frail as those presenting with a fragility hip fracture and therefore there may be a scope to consider muscle sparing approaches in this cohort. Lastly this higher group of frail patients would generally have more post operative complications due to their frailty and these secondary conditions may influence their potential to rehab.

5.8.2 Clinical frailty and objective measures of function

5.8.2.1 BADL

In this study we demonstrate all frailty groups do not return to their objective measures of muscle performance. Significant differences were observed between CFS 4,5,6 with CFS 7 at day 90 when observing objective measures of muscle function. This is relatable to the RMI which measures muscle function subjectively. CFS 7 is consistent in demonstrating no functional improvement from day 7 to 90. Again, this

highlights patients in this group may not have the functional reserve to improve in function.

5.8.2.2 HGS

The results from this study demonstrate as the frailty score increased the hand grip strength decreased. When comparing hand grip strength at day 7 with admission CFS there were no significant differences measurable between adjacent frailty groups except between CFS 6 and 7. This leads us to infer firstly patients with a CFS 7 have a significantly worse hand grip strength than the other frailty groups. Secondly transitioning one frailty level does not cause a significant deterioration in grip strength, but multiple levels does, and lastly the jump in frailty from moderate to severe may be enough to lead to a measurable difference in grip strength suggesting frailty in this modality of measure to not be linear near the extreme end of frailty.

The poor hand grip in patients with increasing frailty and particularly CFS 7 may be explained due to multiple reasons. Cognition is important in the assessment as this is needed to be able to understand how to perform the test and maximal grip strength is needed to be performed. Patient in the higher levels of frailty may will have reduced strength this is already established in existing literature with regards to sarcopenia in the physical manifestation of frailty.(223) In the initial phases of recovery patients are heavily reliant on the use of their upper limbs to help them pull up onto a frame or push out of a chair and hold walking aids. The timing of undertaking the assessment may have had an impact on their assessment if they have already fatigued following a session of physiotherapy or recently mobilised. Pragmatically the grip strength was completed on the same day but timing had to be flexible due to protected mealtimes, other investigations such as blood test and X-rays, and rehabilitation which had to take precedence over the grip strength assessment. Pre-existing disease in the hand and wrist may also have impacted on hang grip strength results.

To limit the impact of cognition on the HGS assessment it was undertaken 7 days following surgery. It was hoped that this would be sufficient time to allow recovery from general anaesthesia, any post operative delirium, and infection. It is not known

when the optimal time is to perform hand grip strength assessment in hip fracture patients. A measurement before the injury would have been ideal but not practically possible. Measurements on admission may have been confounded by pain caused from the hip fracture. A measurement before discharge may have also been informative however direct comparison between patients would have been challenging as acute length of stay is variable. Some patients did not perform the grip strength assessment as they died whilst an inpatient.

Reduction in muscle mass often coincides with decreased strength(277), but this link is complex and not a linear relationship. Studies highlight that post-hip fracture function is more influenced by muscle strength than mass.(278-280) Visser et al. explored this in 90 women over 65, finding poor grip strength (which is correlated with knee and quadriceps strength in the older individual (281, 282). measured with a dynamometer, was linked to poorer mobility function.(278) However, changes in appendicular lean mass (aLM) weren't notably associated with mobility changes. Older women representing a greater strength loss had a significantly worse mobility function who loss less strength.(278) Wehren et al.'s broader study corroborated these findings, showing grip strength changes were more predictive of daily activity difficulties than total body lean mass changes.(279) Low grip strength has also been shown to be associated with lower functional performance measured by Barthels activity of daily living in patients with hip fracture.(283)

5.8.2.3 TUG

Overall, as the level of frailty score increases the TUG time increases. Between adjacent frailty groups there is no measurable significant difference in TUG time. There is an association with CFS and the ability to perform the test. Most patients with CFS 7 were unable to complete the test, with only 3 participants being able to complete it. The number of patients able to complete the test increased as patients became less frail. Since the corelation between TUG time and ability to compete TUG test with frailty score is similar. In the hip fracture cohort, the ability to perform the test may in itself be a better discriminator for function rather than the time itself, however, this results in a binary classification rather than graded and thus patients

with different frailty scale scores would then be grouped together. When observing the time taken for patients to complete the test who were able to, all but one of the patients exceeded the EWGSOP2 cut off for the definition of sarcopenia and therefore all 'probably' sarcopenic.

Patients with a CFS 7 found the test difficult to complete. The TUG test requires sufficient proximal muscle strength to stand from a seated position and thus at the first part of the test this maybe too difficult to perform in the severely frail patients. As previously discussed, the surgical approach to the hip requires dissection through the abductors of the hip and vastus lateralis, therefore this may have impacted the ability to perform the test in this subgroup of patients. It is difficult to know whether it is simply the frailty or the injury and subsequent surgery which plays the most major role in the ability to perform this test. HGS as a measurer of peripheral muscle strength my also play a role in helping to pull up on a frame to get out of a chair or push from the chair up. Hand grip strength is also useful to help with walking when using walking aids which is necessary in the acute recovery phase and many patients rely on this. Studies have shown lower grip strength to be related to an increased incidence of falls.(284) If grip strength is impaired in the severely frail population, then this may contribute to the failure of being able to complete the test. This was demonstrated in this study where patients with a low grip strength had a greater TUG time. There could be a lack of coordination between peripheral and proximal muscle function to coordinate the required movement from seating to standing. Patients too unwell to undertake the test who are naturally of higher frailty and may also fatigue too quickly as they may not have the cardiovascular functional reserve to undertake the test.

5.8.2.4 VLT

Findings from this study show significant differences in VLT thickness both in the fracture and non-fractured limb between CFS 4 and 7 only, at the extreme ends of frailty.

This supports the use of muscle mass being lower in priority of the diagnosis of sarcopenia behind strength and functional performance.(166)

US VLT was measured once post-op during their admission within the first seven days there would be residual effect of surgery causing swelling in the fractured leg and potential dependent oedema in the nonfracture leg by immobility. However, in this study serial measurements to check the rate of decline was not undertaken due to the research burden as a single operator performed all the ultrasound scans. Ideally a pre-surgery, post-surgery, day 7, then day 90, assessment of VLT would have been beneficial to ascertain changes in muscle size and or thickness along with volume assessment of the muscle. Some patients did not have VLT measured as they died as inpatients before the assessment and again as previously discussed hip fracture patients are not routinely followed up following discharge.

Two studies on body composition changes after hip fracture in the elderly found a 5–6% reduction in total body lean mass within a year, mainly occurring in the first 2 to 4 months.(285, 286) Karlsson et al. observed a 5% decrease in total body lean mass and a 9% decrease in lower extremity lean mass between 10 days and 4 months post-fracture, persisting up to 12 months.(285) Fox et al. studied 205 older women, noting a 6% loss in total body lean mass between 10 days and 2 months, stabilizing between 2 and 6 months with a slight increase thereafter. Factors such as age, fracture type, surgery, and body mass index had minimal impact on lean mass loss within the initial 2 months.(286) Another study by D'Adamo et al. found no significant lean mass changes between 3 and 10 days post-fracture in 155 participants.(287) However, research on older adults undergoing joint replacement surgery showed substantial muscle loss within the first week post-surgery, detected using CT scans, suggesting early intervention within 10 days post-surgery and ongoing treatment for at least 2 months during rehabilitation is crucial.(288)

5.8.3 Post operative complications, discharge home, and survival.

This study showed there was no significant correlation between CFS and development of at least one post operative complications in this cohort, however studies have shown that as frailty increases the complications increase.(102) There was also no significant correlation of any other objective measure of muscle assessment with post

operative complication. This could have been due to patient numbers recruited to the study, also established care pathways and clinical guidelines have improved the care of patients with hip fracture through skilled anaesthesia, specialist orthogeriatric input and early surgery.

Discharge home.

Sub analysis of the patients admitted from their own home show, the CFS was negatively weakly associated with new institutionalisation. Discharge back home was associated with day 7 EQ5D, RMI, BADL, CFS, low grip strength according to EWGSOP2 definition, ability to perform the TUG test, and TUG time. The strongest correlation was with the ability to perform the TUG test.

Binary logistic regression and ROC analysis demonstrated two factors predicting discharge home, ability to perform the TUG test and the BADL score at day 7. Both these tests were conducted following the first week after hip fracture surgery. It is reasonable to assume that to be able to get home and function with activities of daily living, the ability to perform coordinated functional movements is paramount, such as required for self-care e.g. getting on and off the toilet seat or chair. Some of these movements are assessed as part of the TUG test. The BADL assessment contains questions which include the activities of daily living such as mobility, self-care, and feeding and would also explain the usefulness of this assessment to facilitate the prediction of discharge home with or without a package of care and facilitate rehabilitation beds. The EQ-5D, GFI and RMI may have been affected by the psychometric portions of their assessments and has been previously demonstrated all decrease during the initial phase of inpatient stay. Observing the individual components of the scoring systems fell outside the remit of this PhD but would be interesting to investigate further.

30-day Survival

As the CFS increases the survival decreases. There is not a significant difference between frailty scores 4 and 5, and 6 and 7. In the first 30 days there is a severe decrease in survival with those with a CFS 7. Previous literature has shown that

survival is linked to frailty(11, 106). Although the NHFS has been shown to be a validated tool for the prediction of mortality at 30 days and 1 year in this cohort of patients it did not show a statistical significance. Kaplan-Meier survival curves shows that patients with both low grip strength and inability to perform the TUG test has a significantly worse survival than having any combination of either a normal grip strength with inability to perform TUG test, low grip strength and able to perform TUG test or normal grip strength and able to perform TUG test. This signifies the combination of the two, proximal and peripheral muscle function and weakness, leads to a worse survival. Interestingly patients with low grip strength and unable to perform the TUG test were predominantly CFS7.

The ability to perform the TUG test, grip strength and day 7 BADL are also good predictors of 30-day mortality and when compared with CFS and NHFS appear to have a greater sensitivity and specificity. However, they would need to be validated in a prospective cohort. The different cut offs for BADL at day 7 could be used for both discharge home and 30-day survival prediction. The next step is to understand the biological mechanisms underpinning poor performance in the frail cohort and thus leads onto the next chapter of gene expression in the skeletal muscle of vastus lateralis.

5.8.4 Reflection and Limitations

This is a unique study which investigates in depth the subjective and objective measures of muscle performance in a range of frail patients following hip fracture. Frailty has been assessed by a clinician trained in the use of CFS, without the time constraints of the admitting orthopaedic team in a busy major trauma centre. Therefore, a comprehensive assessment of frailty has been made and accurately recorded. However, it can be inferred that there may be some potential selection bias or generalisability as the person performing the assessment was a study investigator.

This study has compared objective with subjective assessments of frailty through comparison of the CFS and GFI. This highlights the possibility of using the GFI in future

studies to monitor changes in frailty states following orthopaedic trauma. This is especially paramount as patients who sustain a hip fracture are not followed up routinely. However, this would need validation and is outside the remit of this PhD.

This study has also managed to compare subjective and objective measures of muscle performance in frail patients and facilitated the identification of objective measures to help predict survival and successful discharge home. This will enable stake holders to better allocate resources, time, care, and guide the expectations of patient's recovery.

There are some key limitations the EQ5D was not completed on admission. As a quality-of-life assessment, it was felt that this may have been subject to recall bias. The acute burden of trauma may also have influenced any potential assessment if completed as a retrospective assessment. Post-operatively, concurrent illness and complications may have impacted EQ5D at day 7 but this was outside the remit of this thesis.

Elderly patients with hip fracture are not routinely followed up in the outpatient setting, and therefore undertaking an objective CFS score was not possible. Due to the significant mortality of hip fractures a moderate number of participants are not able to complete the day 90 questionnaires leading to attrition bias.

A pragmatic approach had to be undertaken with the timing of objective assessments. Grip strength, ultrasound and TUG tests had to not interfere with established best practice care. Visiting times, protected mealtimes, physio sessions, clinical review and nursing care all had to be respected and take priority so as not to compromise patient care. The completion of the TUG test also was reliant on the physiotherapy team performing this. Although trained to do so it may not have always been the same person performing the test. However, by adopting a pragmatic approach we believe the results would be generalisable and translatable to other institutions nationally.

VLT was not performed on admission and therefore it was difficult to ascertain if the change in thickness between the non-injured and injured hip was due to the hip fracture or post operative swelling. Ideally it would have been better to repeat these measurements on admission pre-surgery and at day 90. This also would apply to grip strength and TUG test.

5.8.5 Conclusion

The monitoring of fragility hip fracture patients via subjective measures of muscle performance, frailty status and quality of life assessments can be achieved either through personal completion or via a proxy. This would help to demonstrate recovery profiles of differing frailty groups. However, the EQ5D health (visual analogue scale) may not be a good measure of global assessment in frail patient with hip fracture.

We have found mobility assessments either completed subjectively or objectively through the RMI and BADL respectively demonstrate similar outcomes over different frailty states. With increasing frailty, the ability to recover decreases, with severe frailty not recovering at all from day 7 post hip fracture surgery. Physical components of frailty measured through grip strength and TUG test can be used to predict survival, and discharge home

6 The association between Vastus Lateralis muscle gene expression and the clinical frailty scale score on hospital admission in elderly patients with hip fracture.

6.1 Chapter Overview

This chapter quantified muscle mRNA expression of genes linked to the regulation of muscle mass and fuel metabolism in the vastus lateralis of elderly patients. These patients have a range of frailty as defined by the Rockwood clinical frailty scale ranging from pre-frail to mild, moderate, and severely frail. Ingenuity Pathway Analysis, analysis of the mRNA expression identified gene functions and predicted cellular events in muscle from biopsy samples taken at the time of hip fracture surgery in both the non-fractured hip and fractured hip. The key findings of this chapter are threefold. There are a significant number of differentially expressed genes in the vastus lateralis of the fractured leg when compared with the non-fractured leg at the time of surgery. Second in the vastus lateralis of the non-fractured leg, there is virtually no difference in the number of differentially expressed genes when comparing clinical frailty scale scores 5 (mild) ,6 (moderate), and 7 (severe) with the control 4 (pre-frail). As a result, IPA is unable to predict cellular events. Thirdly in the vastus lateralis of the fractured leg, there is a significant number of differentially expressed genes with a gradation in expression as the frailty score increases. Therefore, frailty can be stratified based upon gene expression in the injured limb.

6.2 Introduction

In Chapter 1 the effect of sarcopenia as a physical component of frailty was discussed, and the need to establish the underlying biological pathways associated with frailty. The introduction of this chapter includes skeletal muscle, gene expression, the factors affecting gene expression and the current literature surrounding frailty and hip fracture.

6.2.1 Skeletal muscle

There are three major muscle types found in the human body; skeletal, cardiac and smooth muscle. Each muscle types have specific functions, properties, physiology, and pathologies. Skeletal muscle is an organ primarily concerned with movement and posture. Cardiac muscle encompasses the heart which keeps the body alive. Smooth muscle is present throughout the gastrointestinal, reproductive, urinary, vascular, and respiratory systems.

Skeletal muscle is a specialised tissue for contractile function. It compromises 40% of the total human body weight and is comprised of multiple fibres bundled together into a muscle spindle which give it its striated appearance. A single muscle fibre is composed mostly of actin and myosin fibres covered by a cell membrane (sarcolemma).(289, 290) These fibres are the functional unit of the organ leading to contraction and relaxation. The mechanism of contraction occurs through excitation contraction coupling and neuromuscular transduction. Action potentials from nerve fibres of the central nervous system depolarize muscle down the length of the sarcolemma to the innermost fibres through the transverse tubule (T tubule) system.(289, 290) The neuromuscular junction takes an action potential from the nerve which causes the synaptic vesicles to fuse with the presynaptic membrane, this releases a neurotransmitter acetylcholine into the synaptic cleft. This transmits the information across that cleft with a short delay and releases calcium from the sarcoplasmic reticulum. Calcium subsequently activates ryanodine receptors in the sarcoplasmic reticulum to release even more calcium. The calcium floods in from the sarcoplasmic reticulum and binds to troponin located on the actin filaments. The calcium troponin complex then moves the tropomyosin away from the myosin binding sites, which allows the attachment of the actin and generates the contraction. It is already pre-cocked before the binding site is exposed as soon as it is exposed it binds and powers through.(289, 290) The pre-cocking is related to the hydrolysis of ATP.

6.2.2 Skeletal muscle and frailty

In Chapter 1 we discussed frailty, and its assessment, and the effect of frailty in patients with hip fracture. One of the challenges faced by clinicians and researchers is a lack of understanding in the pathophysiology of frailty. Currently it is predominantly measured by outcomes rather than biological factors. For emerging research in frailty, it is key to investigate the physiological characteristics.

6.2.2.1 Frailty and muscle mass loss

An MRI study involving 26 older adults revealed a 6.4% decrease in thigh muscle cross sectional area among frail individuals compared to their non-frail counterparts,(291) and another study showed average quadriceps muscle cross sectional area was lower in frail patients compared to non-frail ones undergoing haemodialysis.(292) However, comparing these studies is challenging due to the different classification systems utilised to diagnose frailty. A study using CT found reduced thigh muscle cross sectional area in frail nonagenarians compared to non-frail ones, providing one of the few direct measures of muscle cross sectional area in the elderly.(293) However reduced skeletal muscle cross sectional area isn't consistently observed when comparing frail and non-frail individuals.(294)

6.2.2.2 Frailty and muscle function loss

Studies have shown frailty impacts muscle function loss. 20% of patients with lower respiratory tract infections experienced functional decline measured through BADL assessment at discharge(295). Compared to age severity of disease and length of stay cognitive impairment and frailty performed better at predicting functional decline.(295) This has also been demonstrated for elderly patients who have undergone elective surgery where 28.2% of patients experienced declined functional status 30-days following surgery.(296) Although frailty was not objectively stratified in this cohort pre-surgery functional status was shown to be a risk factor for poor post operative function. Initial work relating to this thesis has also demonstrated that frailty is correlated to functional decline requiring institutionalisation and this appears to be a linear correlation up to severe frailty.(102) As far as we are aware at the time

of this study, this is the only study to have investigated frailty specifically in hip fracture patients and their outcomes.

6.2.2.3 Mechanism of skeletal muscle deterioration in frailty

Age related mechanisms and sarcopenia have been shown to contribute to reduced skeletal muscle mass, function, and quality. Sarcopenia is widely considered to be a core component of physical frailty suggesting overlap of inferences. However, there is no longitudinal data in humans specifically looking at frailty and mechanisms of skeletal muscle deterioration.

The observable traits of an organism is referred to as a phenotype and can categorised into physiological, morphological and behavioural characteristics.(297) Phenotypes can be influenced by disease and degenerative processes. Sarcopenia is an example where loss of muscle mass, function and performance with age (170) can negatively influence the physiological phenotype of skeletal muscle deterioration leading to an observable functional decline. How disorders differ in their influence of a phenotype helps to provide a better understanding of the pathophysiology of complex conditions such as frailty. Emerging data form studies have shown through molecular analysis of blood and skeletal muscle at the transcriptome level. Three genes have been found to be associated with frailty TSIX, BEST1, and ADAMTSL4 suggestive of a key role of inflammation and regulation of cellular metabolism.(298) Another study has examined the transcriptome of skeletal muscle and found although differences in gene expression were less marked between young and old groups, significant differences were seen between non-frail and pre-frail elderly individuals in genes regulating muscle function MYLK4 and metabolism.(299)

Anabolic resistance

One proposed mechanism contributing to the decline in muscle mass during old age is anabolic resistance. Which is the reduced ability of feeding and/or exercise to stimulate muscle protein synthesis. Studies have shown a reduced muscle protein synthesis response to essential amino acids in older participants compared younger

participants. To minimise age related effects of gut amino acid absorption, stable isotope tracer infusion techniques to assess protein turnover have been used.(63)

Older volunteers exhibit a diminished activation of anabolic signalling proteins, such as mammalian target of rapamycin (mTOR), in response to essential amino acid infusion, suggesting impaired muscle nutrient sensing, rather than nutrient availability, contributes to the reduced muscle protein synthesis. In addition, older men show a reduced muscle protein synthesis response following resistance exercise compared to younger volunteers, with reduced activation of anabolic signalling molecules.(62)

It is unclear if anabolic resistance is more evident in frail older adults compared to those who are non-frail, or if it is aging itself or secondary to age-related factors, such as reduced activity. There appears to be a consensus that deficits in muscle protein synthesis, rather than increases in muscle protein breakdown, are the main contributors to anabolic resistance in the elderly.(300)

Inflammation

Nonobese frail individuals exhibit heightened levels of interleukin (IL)-6 mRNA and protein content in their vastus lateralis muscle in comparison to non-frail individuals.(291) This increase is attributed to proinflammatory cytokines originating from elevated intramuscular adipose tissue present in frail individuals. The connection between intramuscular adiposity and reduced muscle mass and mobility function in frailty could be explained by this intramuscular adipose tissue-inflammatory axis. Inflammatory processes can contribute to muscle loss by potentially exacerbating anabolic resistance through downregulation of signalling. Experiments involving IL-6 infusion into rodent skeletal muscle, mimicking chronic inflammation levels, have demonstrated induction of muscle atrophy.(301) This effect is believed to occur via decreased levels of insulin-like growth factor 1 (IGF-1), as evidenced by studies showing that overexpression of IL-6 exhibits reduced serum IGF-1 levels. Frail individuals with low relative appendicular skeletal muscle mass (RASM) have been

observed to have lower serum IGF-1 concentrations compared to frail individuals with normal RASM.(301)

Inflammation contributes to sarcopenia by inducing apoptosis in skeletal muscle fibres. Chen et al reported the downregulation of miR-532-3p in muscle from sarcopenic adults. This miRNA targets the proapoptotic gene *BAK1* (BCL2 antagonist/killer 1), downregulation was inflammation dependent with NFKB1, a subunit of the transcription factor NF-κB able to bind to the promoter region of miR-532-3p and repress its expression.(302) Inflammation may induce muscle mass loss during frailty, by impairing muscle regeneration and anabolic processes. However, it is unknown whether these muscle-level characteristics are drivers of muscle deterioration in frailty or a consequence of it.

Physical inactivity

With age physical activity decreases.(303) Reduced physical activity plays a significant role in muscle wasting and diminished muscle quality.(304) Ten days of bed rest leads to approximately 1 kilogram of lean mass loss in the lower limbs and a 16% decrease in knee extensor strength among older adults, primarily due to a 30% reduction in muscle protein synthesis.(59) Data from studies involving disuse or extended bed rest of more than seven days shows notable increases in transcripts related to protein breakdown, immune response, and cell death, along with a decrease in genes associated with mitochondrial function and metabolism(305), similar to patterns observed in studies of frail elderly individuals.(306) Additional research underscores how bed rest diminishes skeletal muscle protein synthesis, contributing to muscle wasting and functional decline. Furthermore, prolonged bed rest and illness likely account for the transition of older adults from a non-frail to a frail state upon hospitalization.(307, 308) It is uncertain whether bed rest exacerbates muscle mass loss and functional decline in individuals who are already frail.

6.2.3 Gene expression

The transformation of genetic information from DNA involves transcription, translation and post-translational modification. DNA contains the genetic instructions for all known organisms' development, function, growth, and reproduction. It is a polymer consisting of two polynucleotide chains containing complementary base pairs (adenine with thymine, and cytosine with guanine) that coil around each other to form a double helix.(309, 310) The biological basis of gene expression is a complex process which involves selection of the gene, via a promoter region, transcription through ribosomal RNA to messenger RNA which is then used as a template to manufacture proteins. Simplistically gene expression can be compared to an "on/off switch" that controls when and where RNA molecules and proteins are produced. However, the complete picture is more complex with modification and attenuation of gene expression downstream (following mRNA production) and upstream in the activation of expression. This can be likened to a "volume control" that determines the amount of these substances produced. The process of gene expression is meticulously regulated and significantly varies depending on environmental conditions and cell type. The RNA and protein products of numerous genes can regulate the expression of other genes. Measuring the functional activity of a gene product or observing a phenotype associated with a gene, can be used to determine the location, timing, and quantity of a gene's expression.(309) Simply measuring the presence of mRNA in a cell determines that gene expression is happening and can (via quantitative methods) give an indication of the level of expression but this is a simplistic picture and does not take into account post transcription modifications.

RNA exists in two classes: coding and non-coding.(311) Coding RNA, mainly messenger RNA (mRNA), is synthesized via transcription by RNA polymerase. mRNA, constituting only 1-5% of total RNA, (312) undergoes maturation involving capping, cleavage & polyadenylation, and splicing.(309) These processes ensure mRNA stability and functionality. Splicing, a crucial step, removes introns and joins exons, enhancing gene expression complexity through alternative splicing. Mature mRNA is then exported from the nucleus for translation into proteins.(309)

mRNA comprises three parts: a 5’ untranslated region (5’UTR), a protein-coding region, and a 3’untranslated region (3’UTR). (309) The coding region contains information for protein synthesis through base triplets. Transfer RNA (tRNA), a non-coding RNA, carries specific amino acids needed for protein extension during mRNA translation. tRNA binds to mRNA via a three-nucleotide anticodon sequence, facilitating amino acid chaining on the ribosome.(309) Each mRNA can yield approximately 2800 protein molecules. Post-translation, amino acid chains fold into three-dimensional structures crucial for function; misfolding can lead to inactive proteins, toxic prions, and diseases. Many proteins are directed to various cellular locations, including secretion outside the cell.(309)

6.2.3.1 Regulation of gene expression

It is important to be aware of how gene expression is regulated. Once specific genes have been identified to be important for a particular function these areas of regulation could serve as potential therapeutic targets. The purpose of gene expression regulation is to control when and how much of a gene's product is produced, this is crucial for cell function and adaptation to the environment. Different types of genes have specific regulatory characteristics: constitutive genes are continually transcribed, whereas facultative genes are transcribed only as needed. Housekeeping genes maintain basic cellular functions and are expressed in all cell types, some serving as standards for expression experiments. Inducible genes respond to environmental changes or cell cycle phases. Gene expression can be modulated at various stages, from DNA transcription to protein modification. The stability of gene products, RNA or protein also influences expression levels. Regulation involves molecular interactions altering DNA transcription and RNA translation. Regulation of gene expression can occur in the transcription or translational phase.(309)

Transcription regulation is influenced by genetic, modulation, and epigenetic factors. Proteins interact with DNA to alter transcription levels, with specific binding sites such as enhancers and silencing sites playing key roles. Regulation mechanisms vary from preventing RNA polymerase binding, to acting as activators. Transcription factor

activity is modulated by intracellular signals, altering binding ability and promoting RNA polymerase recruitment. The nuclear membrane regulates factors' presence and structure changes. Environmental or endocrine signals trigger protein modifications, leading to gene expression regulation.(309)

In post-transcriptional regulation nuclear export regulates gene expression by facilitating RNA export before its translation. Transport through nuclear pores is mediated by importin and exportin proteins. mRNA stability is crucial for protein synthesis; degradation, controlled by various factors. It is particularly vital in eukaryotic cells due to mRNA travel distances. RNA stability is enhanced by post-transcriptional modifications like the 5’ cap and poly-adenylated tail. mRNA degradation serves both defence against foreign RNA and as a regulatory mechanism, facilitated by pathways like RNA interference targeting complementary sequences for destruction.(309)

Translational regulation

Direct regulation of translation is less prevalent than control of transcription or mRNA stability but is occasionally used. Inhibition of protein translation is a major target for toxins and antibiotics, so they can kill a cell by overriding its normal gene expression control.

Post-translational modifications (PTMs) are covalent alterations to proteins, akin to RNA splicing, enhancing proteome diversity. Typically, enzyme-catalysed, they can be reversible, like covalent additions to amino acid side chains, or irreversible, such as proteolytic cleavage of the protein backbone. PTMs serve vital functions in cells, including phosphorylation for protein activation and signalling, histone tail modification for transcriptional regulation, and glycosylation in the immune system. Some PTMs trigger others, like ubiquitination facilitating protein degradation, while proteolysis not only breaks down proteins but also regulates biological processes like DNA transcription and cell death.(309)

6.2.3.2 Factors which affect gene expression

Gene expression varies between cell types, such as peripheral nerve and chondrocytes.(313) Expression also changes with cell age such as between mature cells and embryonic stem cells.(314) Some genes are expressed non-specifically at a constant rate and are referred to as housekeeping genes and these genes can act as a check for cell function and quality control of gene expression data.(315) Prior knowledge of variations in background levels of gene expression in resting cells is beneficial when investigating associations between tissue-specific changes in gene expression and a particular phenotype. Depending on the phenotype under investigation, relevant changes in gene expression can occur in a single or multiple cell types. The identification of these cellular-specific pathways provides insights into the biological processes that may contribute to disease processes. Heterogeneity in gene expression within a population of similar cells can be a source of unexpected variation. Variations in gene expression within a specific cell type include cell-cycle stage, the presence or absence of microbes, and differences in the microenvironment. Consideration should be given with sources of variations among similar populations of cells in a tissue when conducting analysis.(316)

Gene expression varies with age, a meta-analysis demonstrated as age increases 56 genes were constantly over expressed and 17 genes under expressed.(317) These changes appear to be gradual (318) and are likely to be caused by damage to the DNA.(319) Any study involving gene expression should include participants age and if any changes in gene expression are found which are known to change with age this should be considered and controlled for in the statistical analysis. Gene expression is varied between sex in whole blood.(320) Depending on the phenotype under investigation if differences are revealed influenced by sex then this would need to be accounted for in the statistical analysis.

Studies have shown variations in gene expression in multiple tissues over a 24-hour period and across different seasons.(321, 322). This has been seen in whole blood, saliva, liver, and heart. Ideally collection should occur at the same time as possible, and the time and date of sample recorded. Environmental factors have been shown

to influence gene expression.(323) Depending on the phenotype under investigation environmental factor data may need to be collected and considered for its impact. Genetic variations influenced by inheritance can affect gene expression and risk for certain diseases.(324)

Depending on the research question and tissue availability, tissue can be obtained from the patient directly or from a biobank. Large repositories of tissue samples are available for research use. Consent is obtained from the participant at the time of tissue collection for research use.

Advantages of obtaining samples from a biobank include a streamlined process for sample acquisition, no need for consent from the donor at the time tissue is ordered, availability of these samples at low cost, and easy accessibility. However, one disadvantage of using samples from a biobank is that the quality of RNA may be compromised. Research showed that RNA stored at −80 °C degrades within 5 years.(325) The tolerance of RNA for freeze–thaw events is low. Before requesting samples from a biobank, researchers should examine the quality control and quality assurance procedures the biobank employs.

6.2.4 Measurement of gene expression

The ability to quantify the level at which a particular gene is expressed within a cell, tissue or organism can provide valuable information. Similarly, the analysis of the location of protein expression is a powerful tool, and this can be done on an organismal or cellular scale. Investigation of localization is particularly important for the study of development in multicellular organisms and as an indicator of protein function in single cells. Ideally, measurement of expression is done by detecting the final gene product (for many genes, this is the protein); however, it is often easier to detect one of the precursors, typically mRNA and to infer gene-expression levels from these measurements. Examining DNA provides a static picture of what a cell or organism may do or become, whether measuring RNA allows us to see what a cell or

organisms is doing right now providing a more useful insight into the biological processes occurring at a specific moment.

There are several methods that can be utilised for measuring gene expression. These all have a range of advantages and disadvantages. For this project whole gene family expression is required from a relatively small samples that can be compared patient to patient. This allows for analysis that is comprehensive and includes all relevant genes and their regulators.

6.2.4.1 Northern blotting

Northern blotting, akin to the Southern blot, analyses RNA instead of DNA. It's employed in molecular biology to study gene expression by detecting RNA (or mRNA). This technique enables observation of cellular regulation in differentiation, morphogenesis, and disease. RNA is separated on agarose gel via electrophoresis and hybridized to a radioactively labelled RNA probe complementary to the target sequence, then detected via autoradiography.(326)

Northern blotting offers advantages such as RNA size detection, observation of alternate splice products, and the ability to measure RNA quality and quantity before blotting. Membranes can be stored for years and re-probed. However, sample degradation and environmental contamination are common issues. Chemicals used can pose risks to researchers. It's time-consuming, requiring ample RNA and being quantitatively inaccurate for low RNA abundance. Quantification involves measuring band strength, resulting in potential inaccuracies. It typically observes few genes and has lower sensitivity compared to RT-PCR, which has become the preferred method for RNA detection and quantification.(316)

6.2.4.2 Quantitative PCR

The polymerase chain reaction (PCR) is a common method for amplifying DNA; for RNA-based PCR the RNA sample is first reverse-transcribed to complementary DNA (cDNA) with reverse transcriptase. This process is termed reverse transcription PCR

(RT-PCR). To amplify small amounts of DNA, the same methodology is used as in conventional PCR using a DNA template, at least one pair of specific primers, deoxyribonucleotide triphosphates, a suitable buffer solution and a thermo-stable DNA polymerase. A substance marked with a fluorophore is added to this mixture in a thermal cycler that contains sensors for measuring the fluorescence of the fluorophore after it has been excited at the required wavelength allowing the generation rate to be measured for one or more specific products. This allows the rate of generation of the amplified product to be measured at each PCR cycle. The data thus generated can be analysed by computer software to calculate relative gene expression in several samples. Quantitative PCR can also be applied to the detection and quantification of DNA in samples to determine the presence and abundance of a particular DNA sequence in these samples. This measurement is made after each amplification cycle, and this is the reason why this method is called real time PCR (that is, immediate or simultaneous PCR).(327)

Estimation errors arising from variations in the quantification method can be the result of DNA integrity enzyme efficiency and many other factors. For this reason, several standardization systems (often called normalization methods) have been developed. Some have been developed for quantifying total gene expression, but the most common are aimed at quantifying the specific gene being studied in relation to another gene called a normalizing gene, which is selected for its almost constant level of expression. These genes are often selected from housekeeping genes as their functions related to basic cellular survival normally imply constitutive gene expression. Quantification of RT-PCR products can largely be divided into two categories: endpoint and real-time. The use of end-point RT-PCR is preferred for measuring gene expression changes in small number of samples, but the real-time RT-PCR has become the gold standard method for validating quantitative results obtained from array analyses or gene expression changes on a global scale.(316)

6.2.4.3 Microarray

Microarray quantifies multiple transcripts in a single experiment through nucleic acid hybridization of cDNA. RNA is extracted, reverse transcribed, and labelled before

hybridization with immobilized probes on a chip. After washing, fluorescent signals on the chip are scanned, proportional to RNA amounts. Analysis software is then used. Advantages include many transcripts can be quantified in a single experiment, low cost, and no need for prior sequence knowledge. Limitations include multiple tissue samples cannot be assessed at the same time, the control and test tissue sample need to be prepared separately increasing time, an increase in variance output, and reliance on specialized equipment for image processing.(316, 328)

6.2.4.4 RNA Seq

RNA-Seq quantifies the levels of various types of RNA in a sample by sequencing the RNA directly and counting the number of sequences. This approach is different from Northern blotting, which quantifies RNA through gel electrophoresis; qPCR, which quantifies RNA through amplification and dye intensity; and microarray, which quantifies RNA through template hybridization and dye intensity. Initially, RNA is extracted and purified, with the type of RNA targeted based on study objectives. Techniques like poly(A) capture and size selection enrich for specific RNA types, while unwanted species like rRNA and globin RNA can be removed. Alternatively, RNA capture probes isolate specific RNA types. The RNA sample is then prepared into a library, fragmented, and ligated to adapter molecules for subsequent amplification. Finally, the adapter-ligated RNA fragments are amplified and sequenced multiple times in parallel.(316, 329)

Massive parallel transcript sequencing detects genomic alterations at single-nucleotide resolution, surpassing microarray technology in quantifying transcripts' dynamic range. It identifies numerous differentially expressed genes and isoforms, mutations, germ-line variations, and splice variants. RNA-Seq outperforms microarray by detecting 30% more differentially expressed genes. However, RNA-Seq is costlier per sample and demands more computational resources and data storage for downstream analyses compared to microarray.(330)

6.2.4.5 Gene expression profiling

To analyse numerous genes simultaneously within a sample at a high throughput, quantitative PCR can be utilised for hundreds of genes. Another method is hybridization microarray, in which probes are contained on a single array or chip to ascertain the transcript levels of each identified gene within the genome of one or more organisms. Tag-based technologies, such as RNA-Seq and serial analysis of gene expression (SAGE), can provide a relative quantification of the cellular concentration of various mRNA.

The open architecture of the tag-based method is an advantage, as it permits precise measurement of any transcript, regardless of whether its sequence is known or undetermined. An alternative technique that generates enormous quantities of sequence data that can be compared to a reference genome is next-generation sequencing (RNA-Seq). While demanding in terms of time, money, and resources, next-generation sequencing has the capability to detect novel genes, splice variants, and single-nucleotide polymorphisms. Additionally, it can be employed to profile gene expression in organisms where sequence information is scarce or non-existent.

6.2.5 Gene expression in skeletal muscle in patients with hip fracture

During the period of this study other novel work not considered in the introduction have been published. These are briefly summarised below however, none of these studies have shown how the gene expression relates to clinical frailty.

Kang et al in 2021, reported the RNA sequencing profile based on the absence or presence of sarcopenia in elderly osteoporotic female patients with hip fracture. They included patients above 65 years undergoing hip fracture surgery and excluded high energy trauma and non-osteoporotic hip fracture. Patients were assessed and divided into two groups those with and without sarcopenia according to AWGS definitions using grip strength and DXA. Tissue samples for gene expression were obtained from gluteus maximus at the time of surgery during the surgical approach and stored at -80degC. Gene analysis was undertaken using TruSeq Standard mRNA sample

preparation kit and gene expression counts supplied to Deseq2 to extract the genes as differentially expressed genes that discriminate between sarcopenia and non-sarcopenia. For the classification of candidate genes from differentially expressed gene analysis, public databases Gene ontology and Kyoto Encyclopedia of Genes and Genomes were used. A total of 11 samples were analysed from both sarcopenia and non-sarcopenia groups. There was no statistically significant difference in the demographics of patients between the sarcopenia and non-sarcopenia groups. Fifteen differentially expressed genes were identified RUNX1, NGFR, CH3LI, BCL3, PLA2G2A, MYBPH, TEP1, SEMA6B, CSPG4, ACSL5, SLC25A3, NDUFB5, CYC1, ACAT1, and TCAP. The expression patterns of the differentially expressed genes were classified into three groups. Group 1 showed elevated expression in the sarcopenia patients, while the non-sarcopenia patients exhibited low expression levels. Group 2 showed elevated expression in all the sarcopenia patients compared to the low expression in non-sarcopenia patients. Group 3 showed elevated expression in non-sarcopenia patients.

For the functional annotation, the DEGs were classified by the Kegg Brite database. The following functions were identified muscle differentiation capacity and inflammation related genes, skeletal muscle fibre, axon and adipose tissue related genes, mitochondrial energy metabolism related genes.

The limitations of this study are highlighted firstly by the small number of samples in the RNA sequencing and the parameters between muscle and bone interaction may not have been adjusted. The authors also highlighted that it is difficult to rule out the possibility that the results of the study are not due to the disease itself but due to the individual differences, but they did try to overcome this problem by matching demographic factors and test results matched, and PCR verification was conducted.
This study was completed using the AWGS definitions of sarcopenia which would not be relevant for the population of the United Kingdom due to the different cut off values. (331)

Schluessel et al in 2023 recruited 33 participants aged 60 years and older who underwent hip fracture surgery. They excluded patients younger than 60, with specific neuromuscular diseases (myasthenia gravis, muscular dystrophy, amyotrophic lateral sclerosis, polio and myositis) dementia and chronic inflammatory diseases (Crohns and ulcerative colitis, rheumatoid arthritis) glucocorticoid therapy and cancer treatment within 5 years. Patients were divided into a sarcopenia and non-sarcopenia group according to the EWGSOP2 criteria using BIA and Hand grip strength. Tissue samples were obtained from vastus lateralis during the hip fracture surgery near the attachment of the greater trochanter. Tissue was directly cryconserved where tissue blocks mounted on glass slides at -30°C air dried for 2 hours and then stored at -80°C. qPCTR analysis was undertaken using Taqman assay and the relative gene expression of specific genes NR3C1, HSD11B1, FBX032 and TRIM63 were calculated using the 2-delta delta Ct method using 18srRNA as the reference gene. Male and female patients were included, and the only difference found in the characteristics of the participants was the severity of sarcopenia seemed to affect men more than women. No significant difference was found in gene expression profiles between HSD11B1, NR3C1, FBX032 and TRIM63 between sarcopenic and control groups. However, correlations were found between HSD11B1 and skeletal muscle index in the sarcopenic group. However, this study was limited in its sample size and limited investigation into gene expression with only a few genes being selected. Samples although cryconserved appear to be at different temperatures. Gene databases were not used to quantify gene expression into cellular function.(332)

From review of the literature there is an absence of data in relation to the gene expression and cellular function of skeletal muscle and how this relates to frailty and hip fracture between the non-fractured and fractured hip.

6.3 Aims and objectives

6.3.1 Aims

The aim of this study was firstly to quantify the expression of mRNAs representative of a number of cellular functions relevant to muscle homeostasis in the muscle of a

non-injured limb vs an injured limb in all patients to ascertain the impact of injury on muscle gene expression. Secondly, we aimed to consider the same measurements in each limb independently but stratified against frailty status to ascertain the impact of injury on muscle gene expression and frailty status in each limb. We hypothesised that any genes and cellular functions identified as being altered across frailty states would help better characterise frailty in the older person and how it might relate to functional and clinical outcomes following hip fracture surgery. Identification of the genes and cellular functions in relation to frailty status would also facilitate research and identify potential pharmacological approaches to improve patient outcomes.

6.3.2 Objectives

6.3.2.1 Primary Objectives

To quantify the expression of mRNA representative of a range of cellular functions relevant to muscle homeostasis in the vastus lateralis of non-injured and injured limb.

To consider the mRNA expressed in each limb independently but stratified against frailty status.

6.4 Methodology

6.4.1 Muscle biopsy

The muscle biopsy was obtained via the Bergstrom technique which has been previously described in chapter 4.3.2.1 Muscle samples. Analysis of muscle samples were from patients recruited from the FEMUR study as described in chapter 4, who had bilateral vastus lateralis muscle samples.

6.4.2 Tissue management & Quality Control

RNA is a single-stranded molecule and is susceptible to fragmentation by ubiquitous RNase. Care must be taken to preserve RNA during and after sample collection. In the past, because of the RNA degradation that occurred, researchers did not use formalin-fixed paraffin-embedded (FFPE) samples for gene expression studies. In this method,

fresh tissue samples that are to be stored long term for pathology studies or RNA extraction for gene expression experiments were treated with a formaldehyde solution diluted with water and transferred to small containers called cassettes for processing. After processing, the tissue is put in a mould with hot paraffin wax. After the wax cools, the tissue becomes a block. The fixation of these samples with formalin causes cross-linking of nucleic acids with protein molecules, covalent modification of RNA, and fragmentation of RNA transcripts. However, numerous methods are now available to isolate RNA from FFPE samples.(333) Alternatively, biopsies can be frozen without processing. A fresh biopsy should be frozen soon after collection in liquid nitrogen to preserve the RNA (i.e., fresh frozen [FF] sample). In one study, levels of gene expression from FF and FFPE samples showed only 63% concordance. Compared to FF samples, FFPE samples showed lower levels of gene expression.(334)

Quality control

Quality-control procedures to determine the quality and quantity of the RNA is recommended. This is to check for degradation before the start of a gene expression experiment. Processing of degraded RNA in samples can lead to spurious results,(335) where some RNAs may be preferentially degraded while others are stable.(336) One method to check the quality of RNA is to determine the expression levels of housekeeping genes. a qPCR procedure is performed, and fluorescent signal intensity values are evaluated for housekeeping gene transcripts. mRNA stability of housekeeping genes can be affected by cell treatment conditions and cell types. Some housekeeping gene transcripts are more stable than others in a specific cell type. It is important to validate the expression level of different housekeeping genes before use to normalize target gene expression. If degradation of housekeeping gene transcripts is detected in a test sample compared to a control sample, then the sample should be eliminated from the study.(337)

6.4.3 Muscle RNA extraction

Targeted muscle mRNA expression was quantified using real-time polymerase chain reaction (qPCR). Due to its high sensitivity and higher sample throughput compared

to traditional PCR, it is one of the most used techniques for mRNA expression quantification. It combines the detection and amplification processes, building on the principles of PCR, resulting in gene amplification following each thermal cycle being determined in “real-time”. The target RNA is first isolated, and then reverse-transcribed into a cDNA template. The thusly formed cDNA template binds in a 5` to 3` direction to its complimentary DNA oligonucleotides. The cDNA is then heated so the cDNA strand can separate, and then cooled to allow annealing of probes and primers to the cDNA template in the presence of DNA polymerase and deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, dTTP). Further steps of heating and cooling therefore allow for exponential amplification of the target amplicon.

Total RNA was extracted from ~30mg of snap-frozen wet muscle by homogenising in TRIzol. (1ml, Invitrogen, Paisley, UK) and glycogen (10µg/µl; Sigma-Aldrich, Dorset, UK) for ~30s using a polytron homogenizer. Homogenates were then incubated for 5min at room temperature following which Chloroform (200µl) was added and vortexed briefly before further incubation at room temperature for 3min. The homogenates were then centrifuged at 12,000g for 15min at 4°C. These centrifuged samples thus comprised three layers. The clear upper aqueous layer containing RNA was transferred to a new Eppendorf tube. Using iso-propanol, isolated RNA was precipitated (500µl, Sigma-Aldrich, Dorset, UK) and incubated for 20min at 4°C. After incubation the samples were centrifuged for 15min at 12,000g at 4°C to pellet the isolated RNA, the supernatant removed, and the isolated RNA pellet air-dried. Once dry, the pellet was washed in 1ml of 75% ethanol, briefly vortexed and then centrifuged at 7,500rpm for 10min at 4°C. Supernatant was then removed, the pellet was air-dried and then dissolved in 50µl of RNase-free water.

Using a NanoDrop (NanoDrop. Technologies Inc, Wilmingtion, USA), RNA concentration was determined spectrophotometrically by measurement of nucleic acid absorbance at 260nm and 280nm and calculated using Beer- Lambert law (which predicts a linear change in absorbance with concentration). Using the 260:280 ratios,

RNA purity was determined. A value within a range of 1.8-2.0 was considered highly purified RNA.

Muscle RNA extraction was undertaken as per the standard operating procedure SOP038b RNA extraction method for muscle tissue using TRIzol/TRIreagent (Invitrogen/Sigma) (appendix 9.2.1.1)

6.4.4 First strand cDNA synthesis

After spectrophotometric quantification, 1µg of total RNA was incubated in 1µl dNTPs (Promega U1240) and 2µl random hexamers (Promega C1181). The final volume was made of 13µl dNTPs (Promega U1240) using RNase-free water. To denature the RNA secondary structure, samples were heated for 5min at 65°C in a PCR thermocycler, and then immediately placed on ice. By adding 4µl Enzyme buffer, 1µl DTT, 1µl RNase inhibitor (Promega N2511) and 1µl SuperScript^{TM} III reverse transcriptase (Invitrogen Ltd, Paisley, UK), first strand cDNA was created from the RNA and then processed in a PCR thermocycler using heating stages of 25°C for 5min, 50°C for 60min and lastly 70°C for 15min. Samples were then stored at -80°C until further analysis.

First strand cDNA synthesis was undertaken as per the protocol SOP013b First strand cDNA synthesis (appendix 9.2.1.2)

6.4.5 Microfluidic gene card design and protocol

DNA amplification quantification following each thermal cycle can be measured by a variety of different probes with different chemistries. Micro fluidic gene card design and processing was undertaken as per the standard operative procedure protocol SOP027 Microfluidic gene card design and protocol (ABI) (appendix 9.2.1.3)

Multiple mRNA expression measurements (191 targets appendix 9.2.2.1) were made according to the manufacturer's instruction using 100ng of cDNA using Applied Biosystems, Taqman array card (Thermo Fisher Scientific Ltd. Loughborough, UK), which is a 384-well microfluidic gene card designed to perform up to 384 simultaneous real-time PCR (qPCR) reactions. The mRNA investigated were deemed to be

representative of insulin sensitivity, carbohydrate and fat metabolism, inflammation, and protein turnover according to previous findings from human volunteer studies which included unbiased muscle gene analysis.(273) These studies involved interventions that altered physical activity levels, adiposity and fuel oxidation rates all of which are metabolic and physiological trains highly relevant to a bed rest condition. Validation of the approach included detailed literature searches, and SA Biosciences and IPA database interrogation to confirm targets. The targets also included confirmed calcium-activated/sarcoplasmic reticulum genes and further analysed by Applied Biosystems RQ manager software (Thermo Fisher Scientific Ltd. Loughborough, UK) where the threshold level was normalised across all plates before Ct values were calculated for each gene target and sample. Relative quantification of mRNAs of interest were measured using the $2^{-\Delta\Delta Ct}$ method with 18s and Hydroxymethylbilane synthase (HMBS) housekeeper genes used as the endogenous control with the mean of the baseline sample used as the calibrator. To associate a biological function to the identified probe sets, Ct values were uploaded to IPA software (Redwood City, CA, USA) for pathway analysis and gene expression.

Taqman probes consist of a 3’ minor groove binder (MGB) non-fluorescent quencher (NFQ) short oligonucleotide, and a 5’ reporter dye (6-carboxylfluorescein; FAM™). The 3’ quencher and the 5’ reporter are covalently attached to the short oligonucleotide. The presence of a 3`quencher probe in close proximity to the fluorescent 5`reporter dye in the Taqman probe prevents the excitation of the latter, as the energy for excitation is transferred to the 3’ quencher probe by Fluorescent Resonance Energy Transfer (FRET). But after the thermal separation of cDNA, the Taqman. probe containing the 5’ reporter and 3’ quencher anneals to the cDNA strand. In the polymerization phase, the attached 5’ reporter and 3’ quencher are cleaved by DNA polymerase, which through its exonuclease activity liberates the quencher and reporter probes from the oligonucleotide. This allows the reporter probe to be detected spectrophotometrically in response to excitation by a light source following each thermal cycle, allowing for determination of DNA amplification after every cycle. In the early thermal cycles, the end products cannot be quantified, as the intensity of the reporter dye is lower than background fluorescence levels. Once the intensity

exceeds this background fluorescence (Ct threshold), quantification can occur until the plateau phase. If a specific sequence of DNA is expressed to a high degree, amplification of that sequence is noted in earlier thermal cycles.

6.4.6 Quantification of mRNA expression

ABI PRISM 7900HT sequence detection system was used to quantify mRNA expression, and data analysed using SDS 2.1 software (Applied Biosystems, USA). Data were further analysed using RQ Manager software (Applied Biosystems, USA), where the threshold level was normalised across all plates before Ct values were calculated for each gene target and sample. By the comparative Ct method, relative mRNA expression is quantified in relation to the housekeeping gene. Hence choosing a suitable housekeeping gene which does not change with the intervention is key to interpreting gene expression data. Housekeeping genes that are ordinarily chosen for studies are those which are universally expressed and transcribed at a relatively constant rate e.g. (GAPDH; Glyceraldehyde 3-phosphate dehydrogenase), 18s and HMBS (hydroxymethylbilane synthase), in this study the latter two were used. Using the 2_{DDCt} method, relative mRNA expression levels were quantified using the participant's baseline sample as the calibrator, and HMBS as the housekeeping gene (endogenous control), from the following formula:

$$2^{\Delta\Delta Ct} = \Delta Ct_{\text{target gene baseline}} - \Delta Ct_{\text{target gene baseline}} - \Delta Ct_{\text{target gene post-intervention}},$$

$$\text{Whereby: } \Delta Ct = Ct_{\text{target gene}} - Ct_{\text{housekeeping gene}}$$

6.4.7 Ingenuity pathway analysis

Muscle mRNA abundance was analysed using Ingenuity Pathway Analysis (IPA) software (Redwood City, CA, USA). IPA is a commercial web-based software application that allows for integration, analysis and understanding of data obtained from gene expression, SNP and miRNA microarrays; and proteomics, metabolomics and RNA sequencing experiments. It can be implemented towards small-scale experimental analysis that generates chemical and gene lists. Interpretation and analysis of data by IPA is based on a manually curated and comprehensive database (Ingenuity Knowledge Base, https://analysis.ingenuity.com/). Regulators,

mechanisms, relationships, pathways and functions pertinent to changes noted in an analysed dataset are identified by powerful algorithms. IPA analysis therefore integrates expressed genes and infers the likely biological effect of the whole pathway. This enables the understanding of experimental results in the context of biological systems.

For many experiments, especially high throughput experiments it can be very difficult to interpret data in a broader biological context and conclude how all the different pieces of information fit together. Significant challenges include reading and interpreting all the relevant publications which exist that are necessary to understand the research. This can be a time-consuming process. IPA is a web-based bioinformatics application that allows researchers to upload data analysis results from high-throughput experiments such as microarray and next generation sequencing for functional analysis integration and further understanding.

The ingenuity knowledge base contains a large amount of information and includes millions of manually reviewed findings from peer reviewed literature, different pathways and system models, public databases, experimental datasets, and customer proprietary data. There are strict quality control procedures to ensure the finding which underly the knowledge base is accurate and timely. In addition to providing this information it is structured to form ingenuity ontology, to provide a combination of knowledge and software algorithms which enables the quick analysis and accurate interpretation of the biological meanings of experimental data. At the end of September 2022, the knowledge base contained 6.9 million findings increasing weekly.

It is also important to appreciate that gene expression changes rapidly over time and as such the results presented represent a ‘moment in time’ simply interpreting individual genes makes this an unreliable process. Including synergists, antagonists, promoters and regulators gives a more comprehensive and accurate picture which is less dependent on the moment of the experiment.

In IPA gene protein or chemical experimental data can be uploaded, of which there are 5 types of core analysis including gene expression, toxicology, metabolomics, variant effects, and phosphorylation. IPA can help to answer the following questions: which signalling and metabolic are enriched in the data, what upstream regulators are significant and how do they affect diseases or functions, which diseases and functions are overrepresented, which molecules are highly connected and demonstrate potential molecular interactions, which upstream regulators and pathways are activated or inhibited, which analyses are similar or dissimilar to my analysis.

Statistics

Analysis of mRNA abundance by IPA firstly analyses the fold change from the mean baseline calculated from the aforementioned Ct values at each time point to determines the differential expression of mRNA. Statistical significance is then determined from the fold change using a paired sample t-test. The data is then filtered with a fold change cut-off of 1.5 and p value <0.05 to select the most significantly changed genes, which are then used as inputs for the subsequent IPA analysis. Z score calculations is also used in data interpretation in two ways. Firstly, it is used to make predictions about the activation or inhibition of pathways, it does require some directional expression or phosphorylation data. It compares the expression or phosphorylation patterns of the molecules within the data set with what is known in the literature in the ingenuity knowledge base. A z score ≥2 (activation) and ≤-2 (inhibition). Secondly, a separate z score is calculated for the analysis match feature between two analyses to identify other analysis which are very similar or dissimilar to the experimental data submitted. Here a z score ≤2 the two analysis have a good match and ≤-2, do not match, and have different biological signatures. The p values are used to calculate significance in all types of analysis whereas the z score is only used in downstream effects, upstream regulators, and analysis match features.

6.4.8 Workflow for IPA analysis

Analysis of mRNA abundance by IPA was performed as follows:

First, fold change from mean baseline was calculated from the aforementioned Ct values to determine differential expression of mRNAs. Subsequently, statistical significance (p-values) was determined from the fold change data for each frailty score using a paired sample t-test. Fold change and statistical significance of all genes are shown in Appendix 8.2.2.9Data filtering was set with a fold change cut-off of 1.5 and p-value cut-off of $p<0.05$ to select for the most significantly changed genes. These genes were then used as the input for core IPA analysis.

The overall outcome of IPA (e.g. upstream regular analysis, cellular function, activation status) was predicted by calculating a regulation Z-score and an overlap p-value, which were based on: (i) the number of regulated target genes' function; (ii) the magnitude of expression change (iii) the direction of expression change; and (iv) their concordance with the IPA database, which is constructed from a curated literature database.

A second p-value called the "p-value of overlap" was calculated by IPA to identify significantly enriched function pathways from the submitted list of significantly changed genes. These p-values were generated from the right-tailed Fisher's Exact Test, and a significance threshold of 0.05 was used to assess the statistical significance of the function pathways. To control for any enrichment of false positive results when undertaking multiple comparisons (type II errors) IPA utilises Bonferroni's corrected p-value < 0.05.

6.5 Results

6.5.1 Demographics of patients with bilateral Vastus Lateralis muscle samples

Observing the participants who had bilateral vastus lateralis muscle biopsies there was no statistically significant difference in age or sex. As frailty scale score increased the mean BMI decreased with the main differences occurring in CFS 7. This also held true for AMTS, residential status and pre-injury mobility as shown in table 6.1.

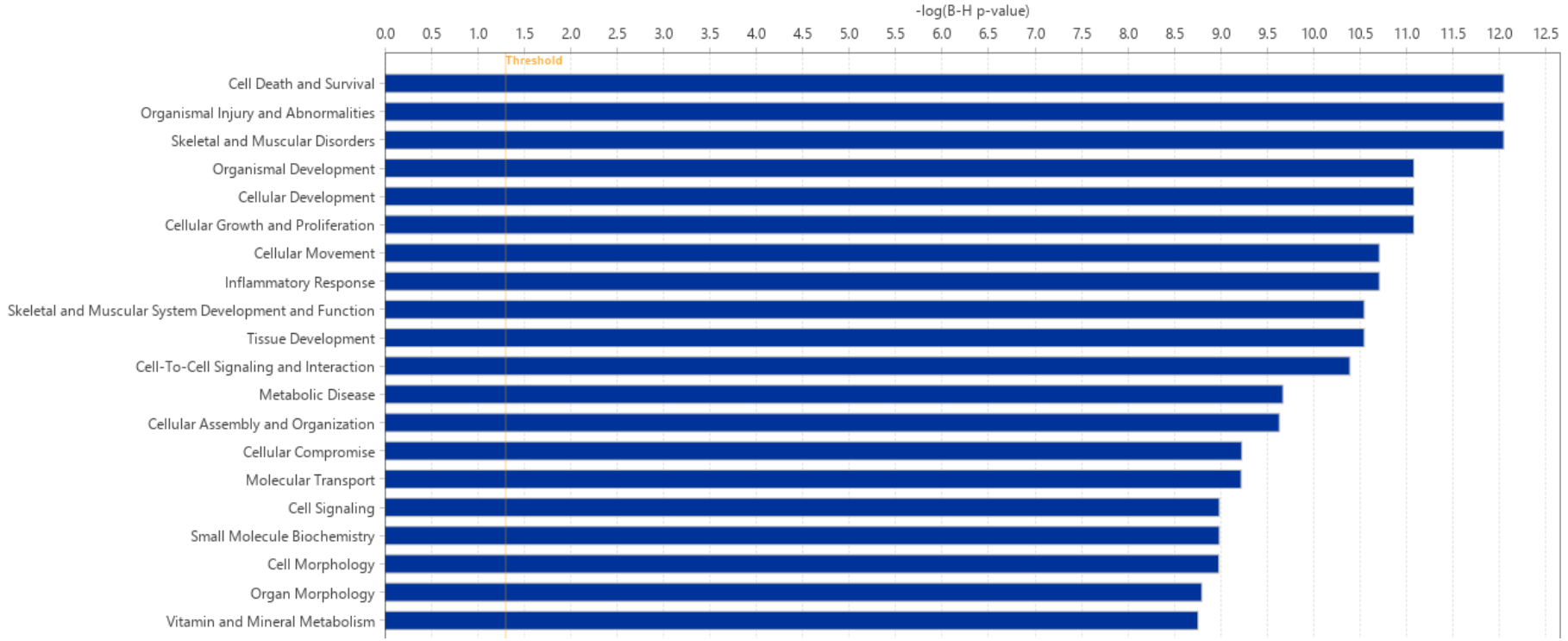
Table 6.1 Demographics of participants who had bilateral vastus lateralis muscle biopsies taken. *ANOVA, +Pearsons Chi squared test

		CFS 4 n=20 (95%CI)	CFS 5 n=20 (95%CI)	CFS 6 n=20 (95%CI)	CFS 7 n=20 (95%CI)	P value
Mean Age Years		83.15 (79.77, 86.53)	86.50 (83.39, 89.61)	87.90 (84.83, 90.97)	87.45 (84.14, 90.76)	0.129*
Mean BMI Kg/m^2		27.33 (24.41, 30.24)	23.17 (20.47, 25.87)	22.91 (21.05, 24.76)	21.07 (19.26, 22.88)	0.002*
AMTS		8.90 (7.91, 9.89)	8.80 (8.24, 9.36)	8.05 (6.71, 9.39)	2.30 (0.93, 3.67)	<0.001*
Sex	Male	7	4	6	1	0.111+
	Female	13	16	14	19	
Residential status	Home	20	20	16	8	<0.001+
	Residential Home	0	0	3	6	
	Nursing Home	0	0	1	6	
Pre-Injury mobility						
Freely mobile without aids		13	7	5	7	
Walks with 1 aid		6	6	4	4	0.027+
Walks with 2 aids / frame		0	7	9	5	
Indoor mobility but never goes outdoors without help		1	0	2	4	

6.5.2 Gene expression in the fractured and control hip (all participants)

Complete gene expression analysis was undertaken in 76 samples in the fractured hip and 77 in the control (non-fractured) hip. Thirty-five genes were differentially expressed when comparing the fractured hip with the non-injured (control) hip. Figure 6.1 depicts the top twenty cellular functions most altered when comparing the fractured hip with the control hip. The x-axis displays the -log of the p values. The p-value associated with each cellular function is a measurement of the likelihood that the association between a set of focus transcripts and a given function is due to random chance. The -log of the p-value was calculated using Fisher's exact test (right tailed) as previously mentioned.

Figure 6.1: Fractured Hip – Top 20 functions as identified by IPA as being altered from the control (non-injured) leg



Cellular functions identified by IPA as being altered from the control hip (n=77) in vastus lateralis muscle in the fractured hip (n=76) during the time of surgery. The x-axis displays the -log of the p value and the y axis displays the cellular functions most affected by the fracture. The p-value associated with each cellular function is a measurement of the likelihood that the association between a set of focus transcripts and a given function is due to random chance. The -log of the p value was calculated by Fisher's exact test right tailed ($p<0.05$). The threshold line corresponds to a p value of 0.05.

IPA detected the most altered cellular functions [-log (p value)>12.0], in descending order of magnitude, were those associated with cell death and survival, organismal injury and abnormalities, and skeletal and muscular disorders.

Figure 6.2 – 6.4 are included by way of example to illuminate precisely how any cellular function identified as being altered in Figure 5.4 was influenced by the fractured hip. Each figure depicts the most differentially regulated mRNAs from the control (outer ring of the rosette), and the cellular events predicted to result from these collective mRNA changes in the fractured leg (icons in the centre of the rosette). The functions selected to be shown here, included those associations with cell death and survival, organismal injury and abnormalities, and skeletal and muscular disorders. In choosing which functions to depict, those were considered that changed the most in each group relative to the control and for which the most network predictions were made with the highest confidence (the most intense colours). Schematics for the rest of the cellular functions which changed are shown in appendix 9.2.2.2 The outer circle of symbols represents the genes involved in the IPA prediction model. The prediction legend depicts the increased or decreased presence of the gene noted by the colour red and green respectively. The intensity of the colour represents a greater increase or decrease in expression. The inner symbols are the functions of the genes either activated or inhibited by the colour's orange and blue respectively. The greater the intensity of the colour the more confidence IPA demonstrates in the prediction model.

Cell Death and Survival (Figure 6.2)

Twenty-five mRNAs were identified as being altered in abundance relative to the control hip. Twenty-one were up-regulated and four were down-regulated. The most up-regulated mRNA from the baseline were MT1A, SOCS3, TNC, and CCL19, while PAX3 ATP2A1, and ATP2B2, were the most down-regulated transcripts. Based upon the collective changes in mRNA abundance from the control, IPA predicted with high confidence inhibition of apoptosis of muscle cells and cell death of muscle cells, and activation of cell viability and cell viability of connective tissue cells.

Organismal Injury and Abnormalities (Figure 6.3)

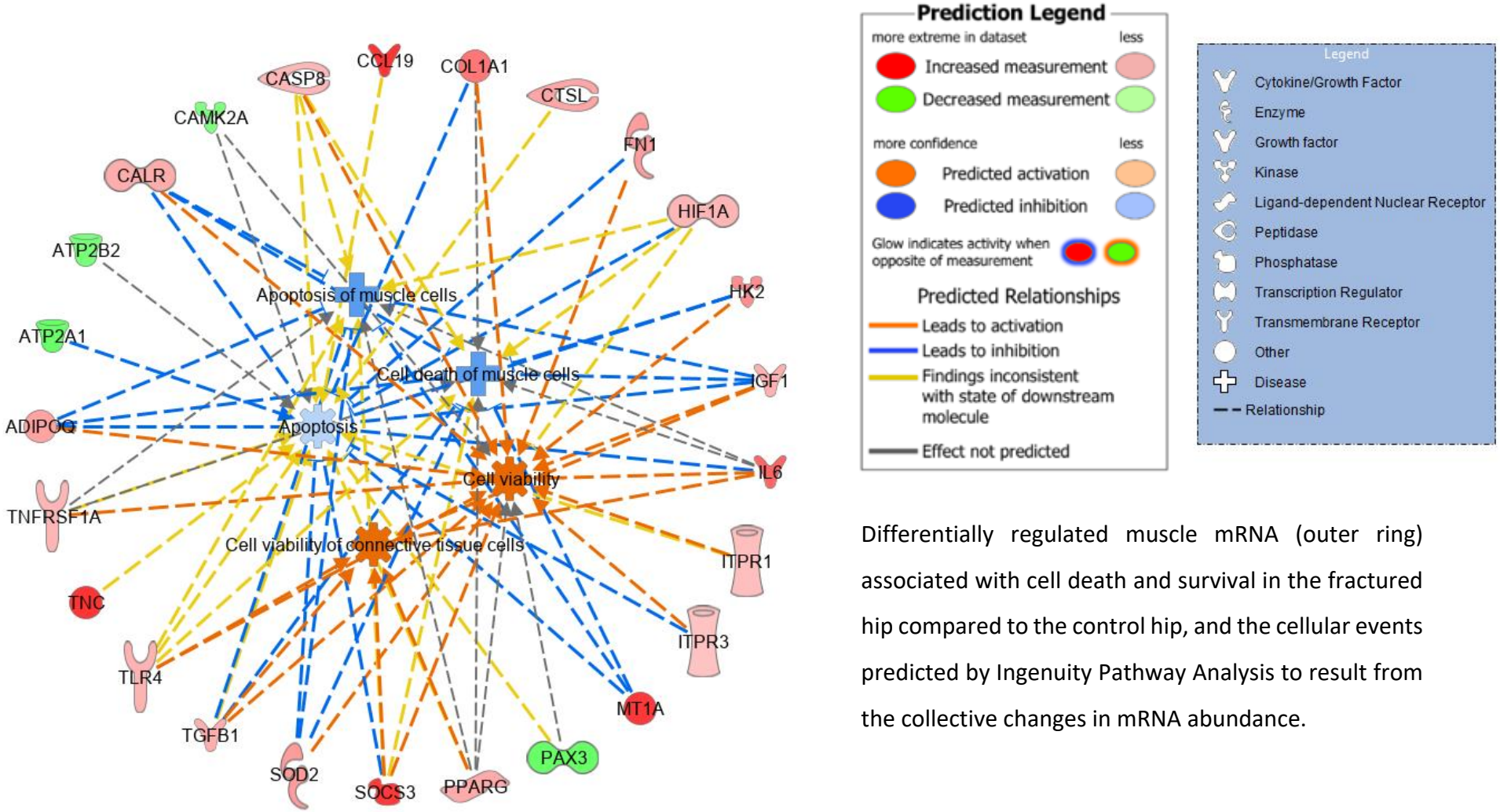
Twenty-one mRNAs were identified as being altered in abundance relative to the control hip. Eighteen were up-regulated and three were down-regulated. The most upregulated mRNA from the baseline were MT1A, SOCS3, and TNC, while MYLK2, and ATP2A1 were the most down-regulated transcripts. Based upon the collective changes in mRNA abundance from the control, IPA predicted with high confidence inhibition of cell death of connective tissue cells, apoptosis of muscle cells, and cell death of muscle cells.

Skeletal Muscle Disorders (Figure 6.4)

Fifteen mRNAs were identified as being altered in abundance relative to the control hip. All were up regulated. The most up-regulated mRNA from the baseline were MT1A, and SOCS3, based upon the collective changes in mRNA abundance from the control, IPA predicted with high confidence inhibition of apoptosis of muscle cells, and cell death of muscle cells.

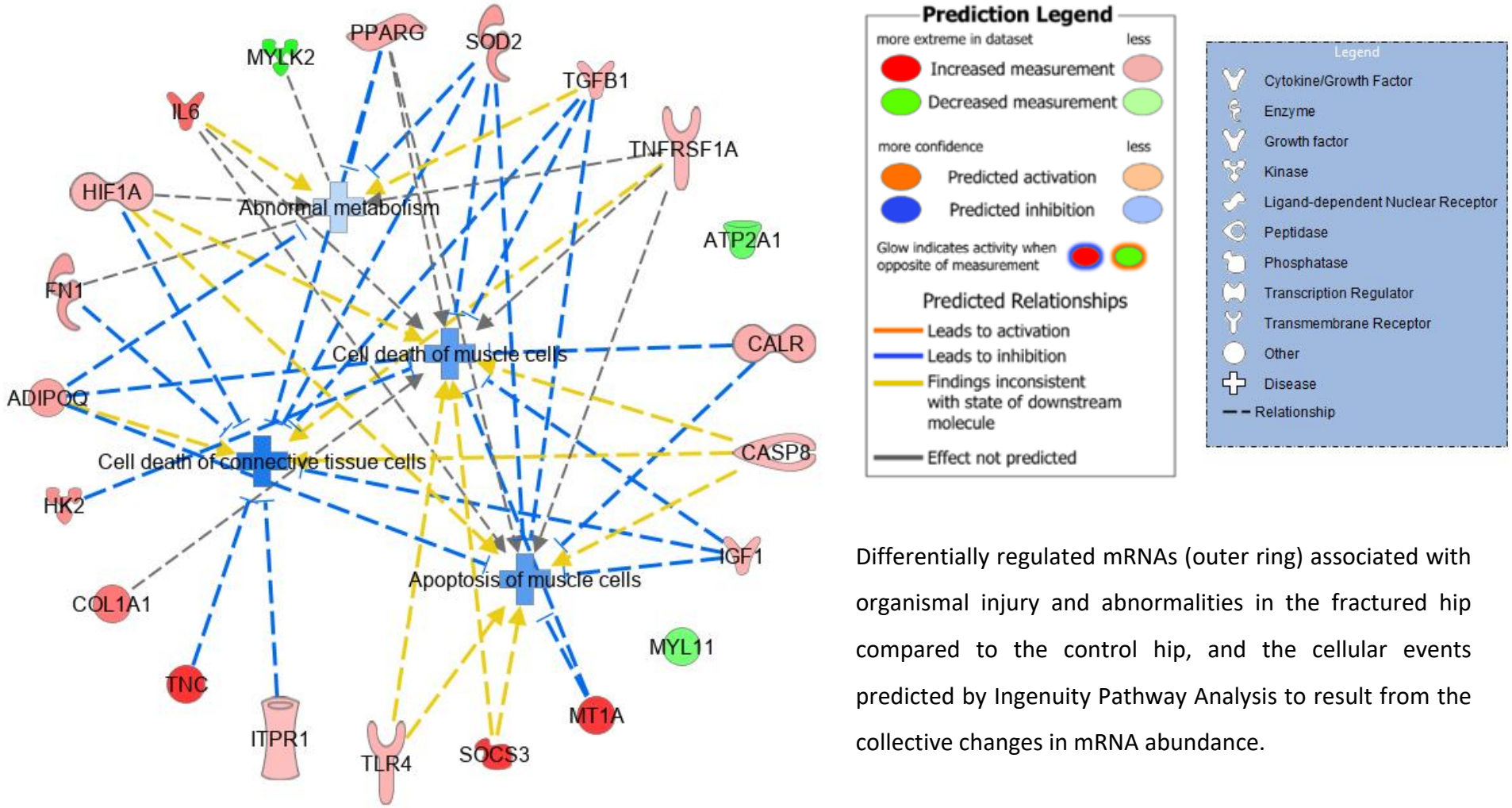
Observing the IPA predictive rosettes the cellular function of inflammatory response also showed a significant network of gene expression demonstrated in Figure 6.5 Nineteen mRNAs expressed, all were up-regulated with TNC, SOCS3, and CCL19, showing the greatest expression. Here cellular functions with a high degree of activation included immune response of phagocytes, aggregation of phagocytes, aggregation of leukocytes, recruitment of phagocytes, migration of phagocytes, inflammatory response. A high degree of inhibition of the inflammation of organ was also observed.

Figure 6.2: Fractured hip – Cell death and survival



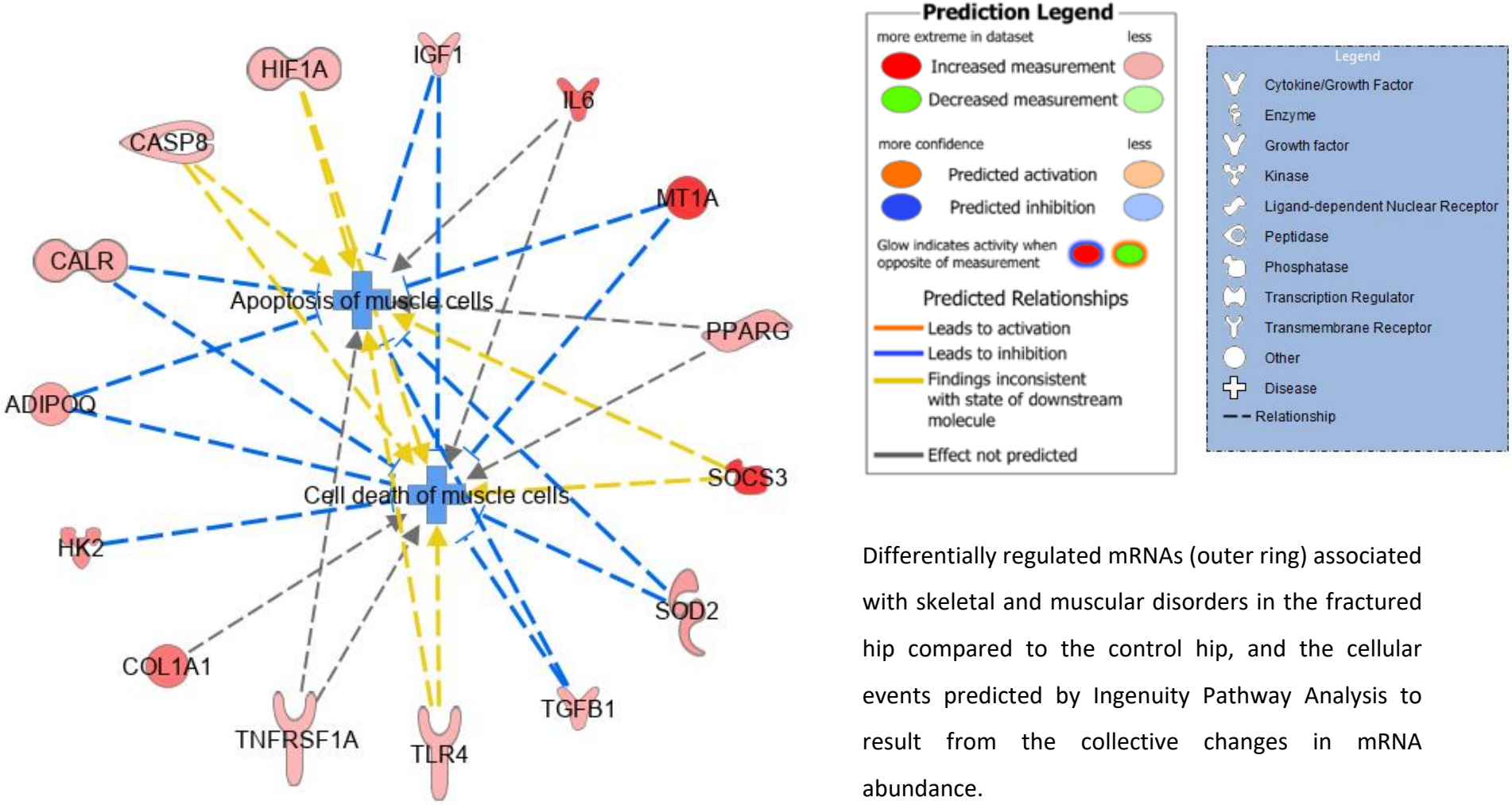
Differentially regulated muscle mRNA (outer ring) associated with cell death and survival in the fractured hip compared to the control hip, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Figure 6.3 Fractured hip – Organismal injury and abnormalities



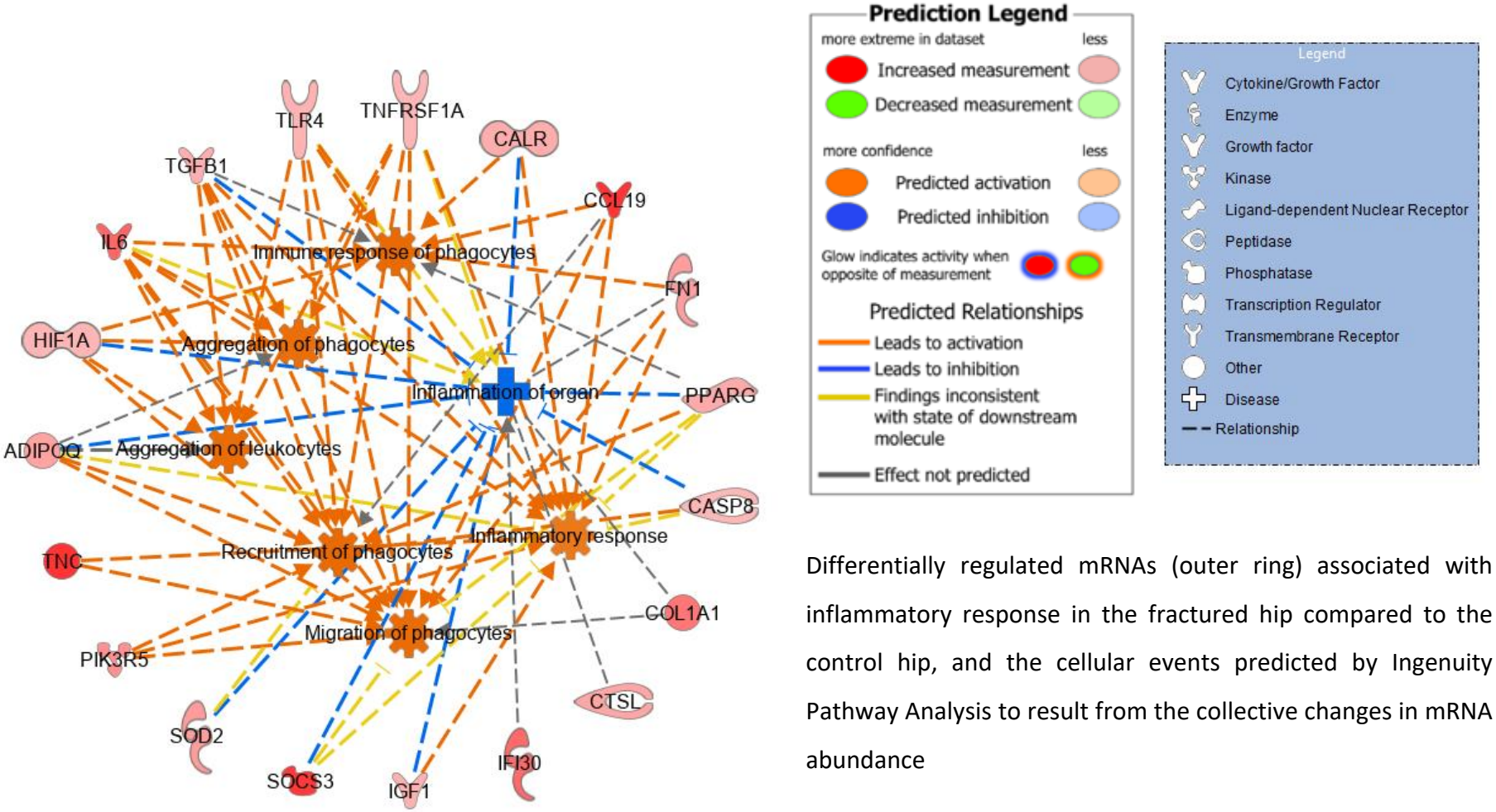
Differentially regulated mRNAs (outer ring) associated with organismal injury and abnormalities in the fractured hip compared to the control hip, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Figure 6.4 Fractured hip – Skeletal and muscular disorders



Differentially regulated mRNAs (outer ring) associated with skeletal and muscular disorders in the fractured hip compared to the control hip, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Figure 6.5 Fractured hip – Inflammatory response

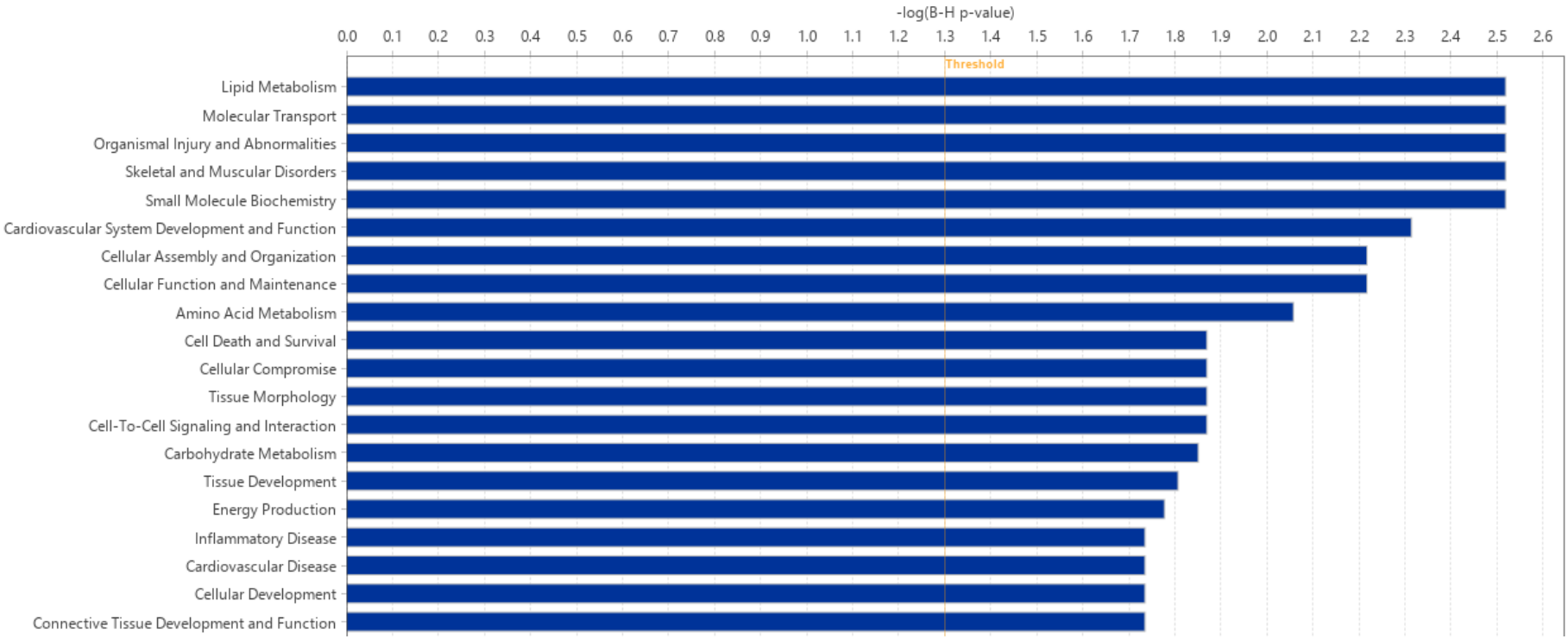


Differentially regulated mRNAs (outer ring) associated with inflammatory response in the fractured hip compared to the control hip, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance

6.5.3 Gene expression in the control hip, and its relationship to frailty status

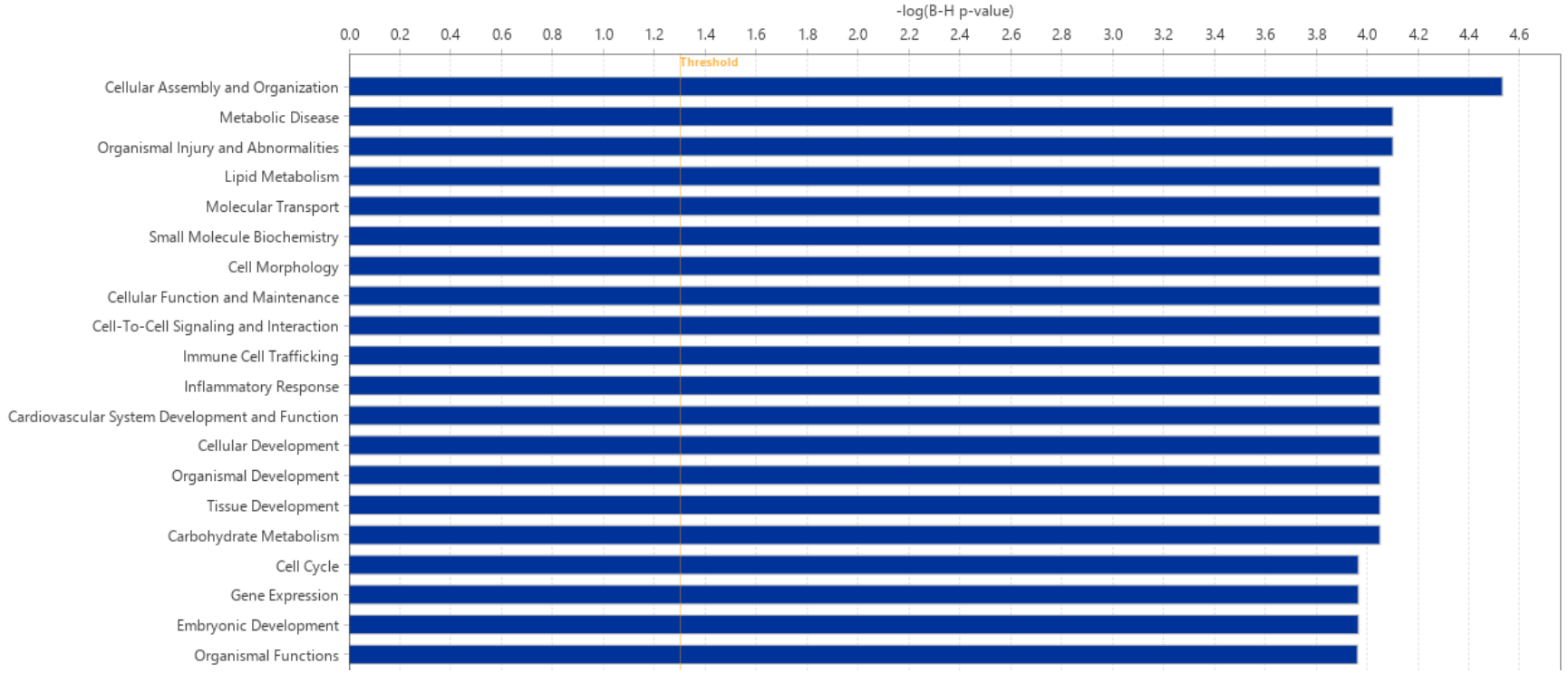
Gene expression and the cellular functions expressed between frailty groups analysis was undertaken firstly in the control hip. Participants who were CFS 4 (pre-frail) were used as the comparator group Figures 6.6–6.8 depicts the most changed cellular functions demonstrating a statistically significant difference between each frailty group and the control CFS4 with their -log p values shown. The x-axis displays the -log of the p values. The p-value associated with each cellular function is a measurement of the likelihood that the association between a set of focus transcripts and a given function is due to random chance. The -log of the p-value was calculated using Fisher's exact test (right tailed). Overall, the -log of the p values for all cellular functions was relatively low. Due to this IPA was unable to gene networks altered between CFS 4 and 5 and CFS 4 and 6. Only two and four genes were significantly differentially regulated respectively. Twelve genes were significantly differentially regulated when comparing CFS 4 (n=19) with CFS 7 (n=19) in the control limb. Again due to the insufficient number of differentially expressed genes IPA was only able to make limited predictions in cellular functions with small gene networks. In short, there was no robust difference in muscle gene expression from CFS level 4 with increasing frailty status in the non-injured limb Schematics for the cellular functions which changed are shown in appendix 9.2.2.4

Figure 6.6 Top 20 cellular functions identified by IPA in the control hip when comparing CFS 5 with CFS 4.



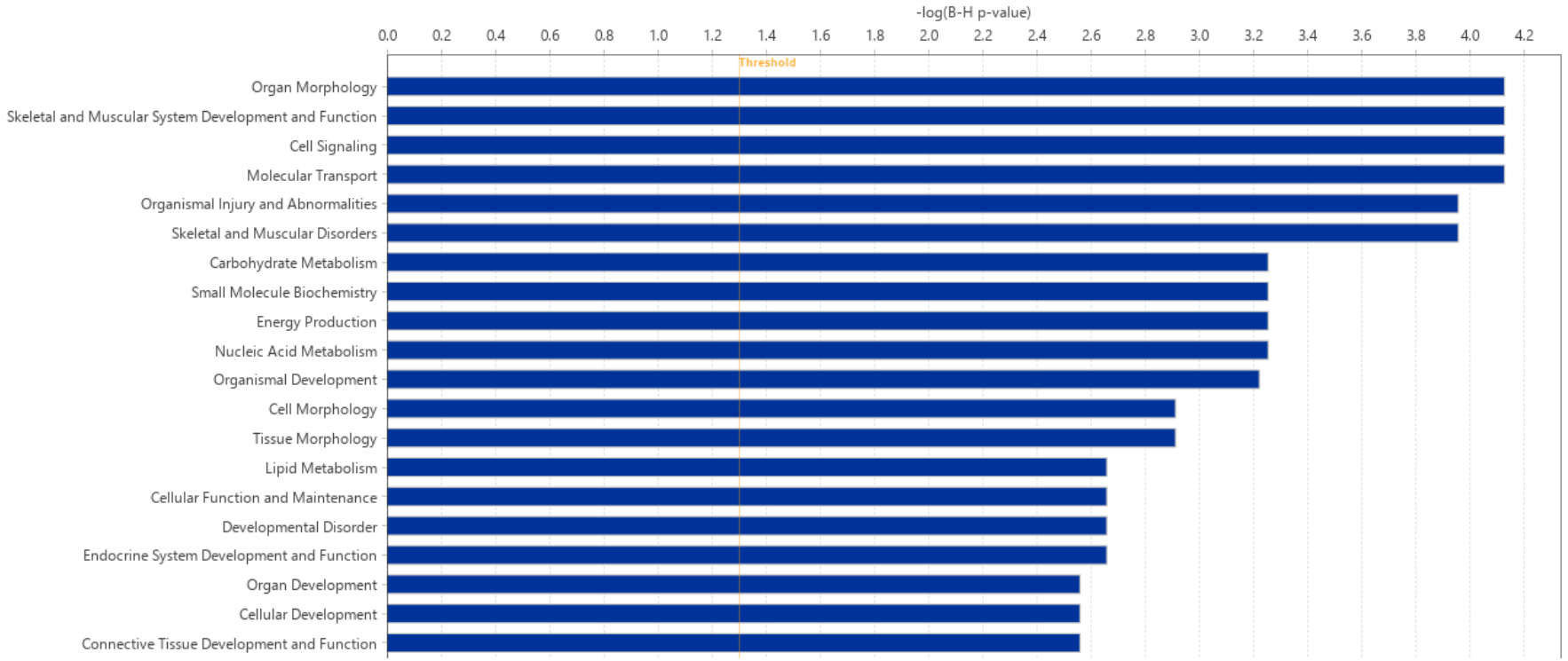
Cellular functions in the non-fractured hip identified by IPA as being altered from the control frailty CFS 4 group (n=19) in vastus lateralis muscle in patients with CFS 5 during the time of surgery. The x-axis displays the -log of the p value and the y axis displays the cellular functions most affected by the fracture. The p-value associated with each cellular function is a measurement of the likelihood that the association between a set of focus transcripts and a given function is due to random chance. The -log of the p value was calculated by Fisher's exact test right tailed ($p<0.05$). The threshold line corresponds to a p value of 0.05.

Figure 6.7 Top 20 cellular functions identified by IPA in the control hip when comparing CFS 6 with CFS 4.



Cellular functions in the non-fractured hip identified by IPA as being altered from the control frailty CFS 4 group (n=19) in vastus lateralis muscle in patients with CFS 6 during the time of surgery. The x-axis displays the -log of the p value and the y axis displays the cellular functions most affected by the fracture. The p-value associated with each cellular function is a measurement of the likelihood that the association between a set of focus transcripts and a given function is due to random chance. The -log of the p value was calculated by Fisher's exact test right tailed ($p<0.05$). The threshold line corresponds to a p value of 0.05.

Figure 6.8 Top 20 cellular functions identified by IPA in the control hip when comparing CFS 7 with CFS 4.



Cellular functions in the non-fractured hip identified by IPA as being altered from the control frailty CFS 4 group (n=19) in vastus lateralis muscle in patients with CFS 7 during the time of surgery. The x-axis displays the -log of the p value and the y axis displays the cellular functions most affected by the fracture. The p-value associated with each cellular function is a measurement of the likelihood that the association between a set of focus transcripts and a given function is due to random chance. The -log of the p value was calculated by Fisher's exact test right tailed ($p<0.05$). The threshold line corresponds to a p value of 0.05.

6.5.4 Gene expression in the fractured hip, and its relationship to frailty status

Gene expression and cellular functions expressed between frailty groups analysis was undertaken in the fractured hip. CFS 4 (pre-frail) was used as a control to compare the frail groups CFS 5-7. Figures 6.9 – 6.11 depicts the top twenty most changed functions demonstrating a statistically significant difference from CFS 4 in each increasing frailty group with their -log p values shown. The x-axis displays the -log of the p values. The p-value associated with each cellular function is a measurement of the likelihood that the association between a set of focus transcripts and a given function is due to random chance. The -log of the p-value was calculated using Fisher's exact test (right tailed).

Comparing CFS 4 (n=19) with CFS 5 (n=20)

IPA detected the most altered cellular functions [-log (p value)>7.6], in descending order of magnitude, organismal injury and abnormalities, skeletal and muscular disorders, and skeletal and muscular system development and function Figure 6.9.

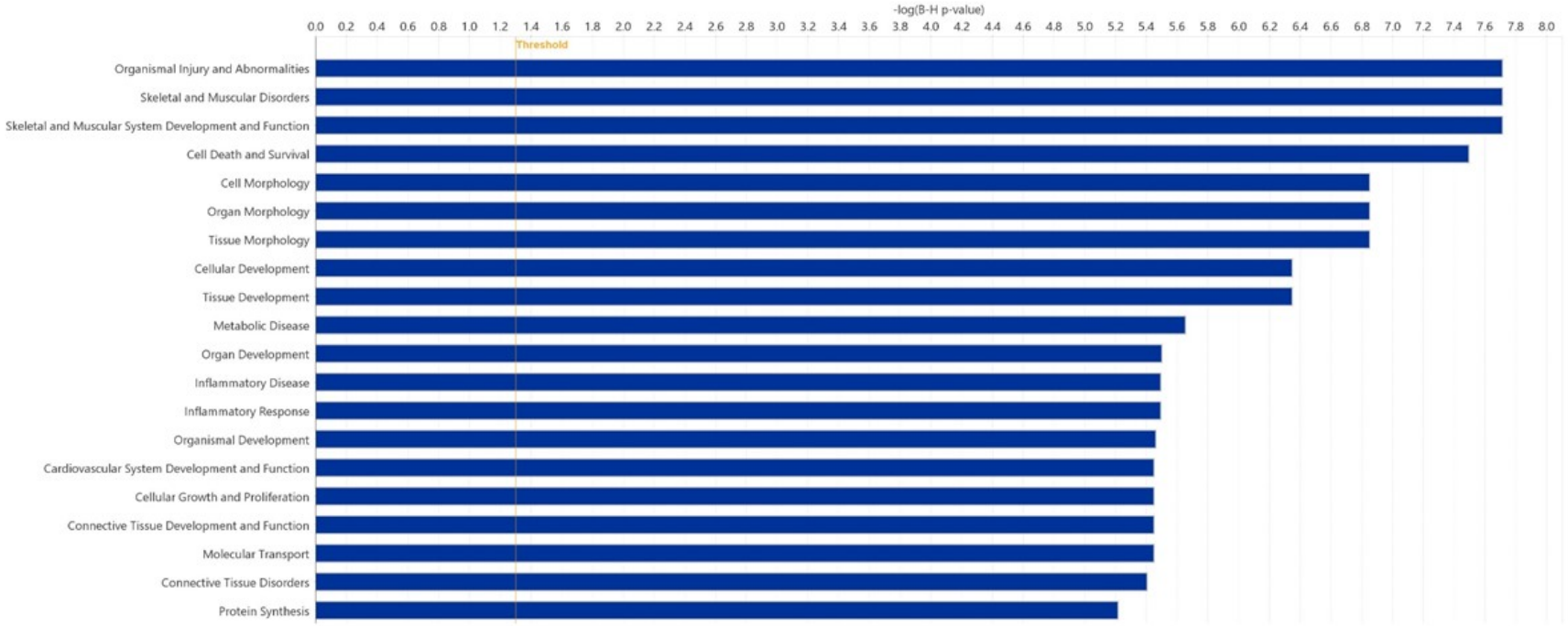
Comparing CFS 4 (n=19) with CFS 6 (n=19)

IPA detected the most altered cellular functions [-log (p value)>6.4], in descending order of magnitude, metabolic disease, organismal injury and abnormalities, cellular movement and inflammatory response Figure 6.10.

Comparing CFS 4 (n=19) with CFS 7 (n=19)

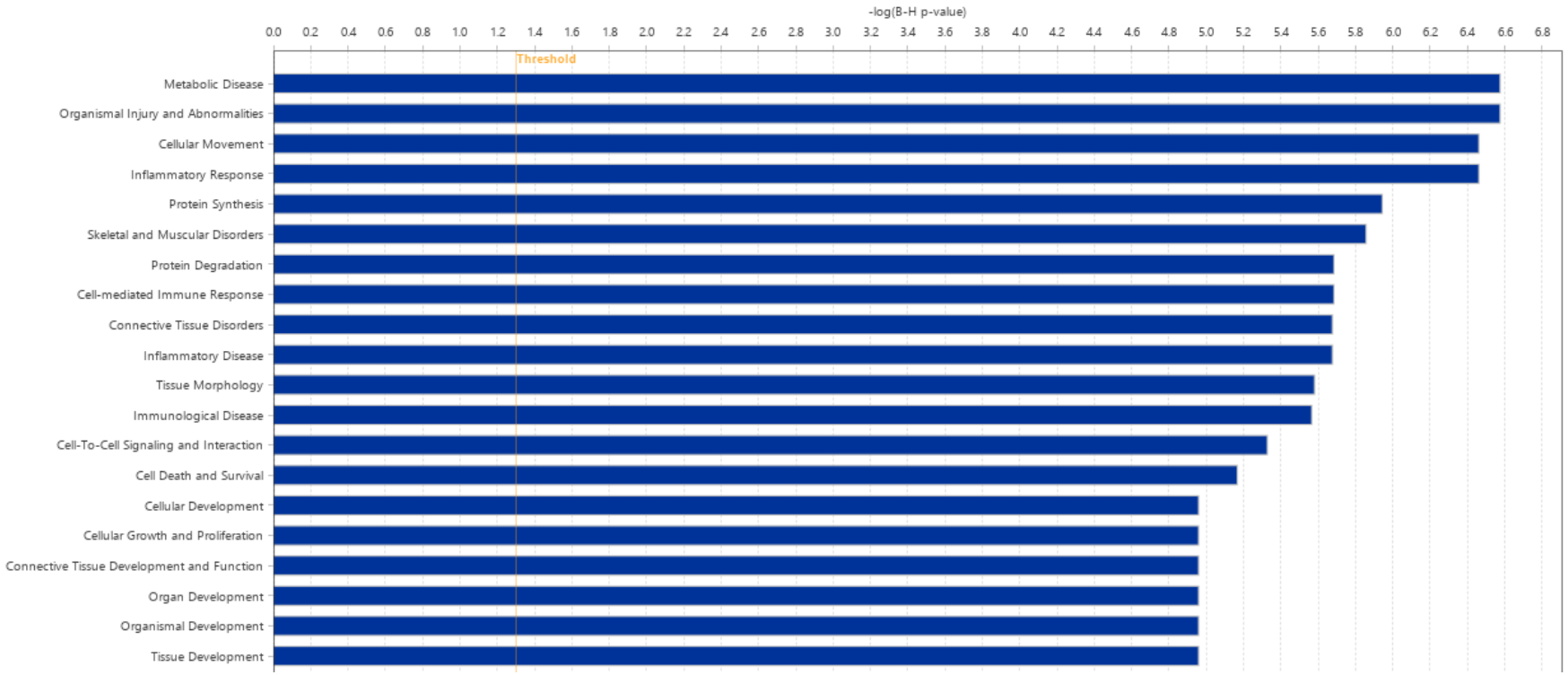
IPA detected the most altered cellular functions [-log (p value)>9.0] in descending order of magnitude, organismal injury and abnormalities, and skeletal and muscular disorders Figure 6.11.

Figure 6.9 Top 20 Cellular functions identified by IPA in the fractured hip when comparing CFS 5 with CFS 4.



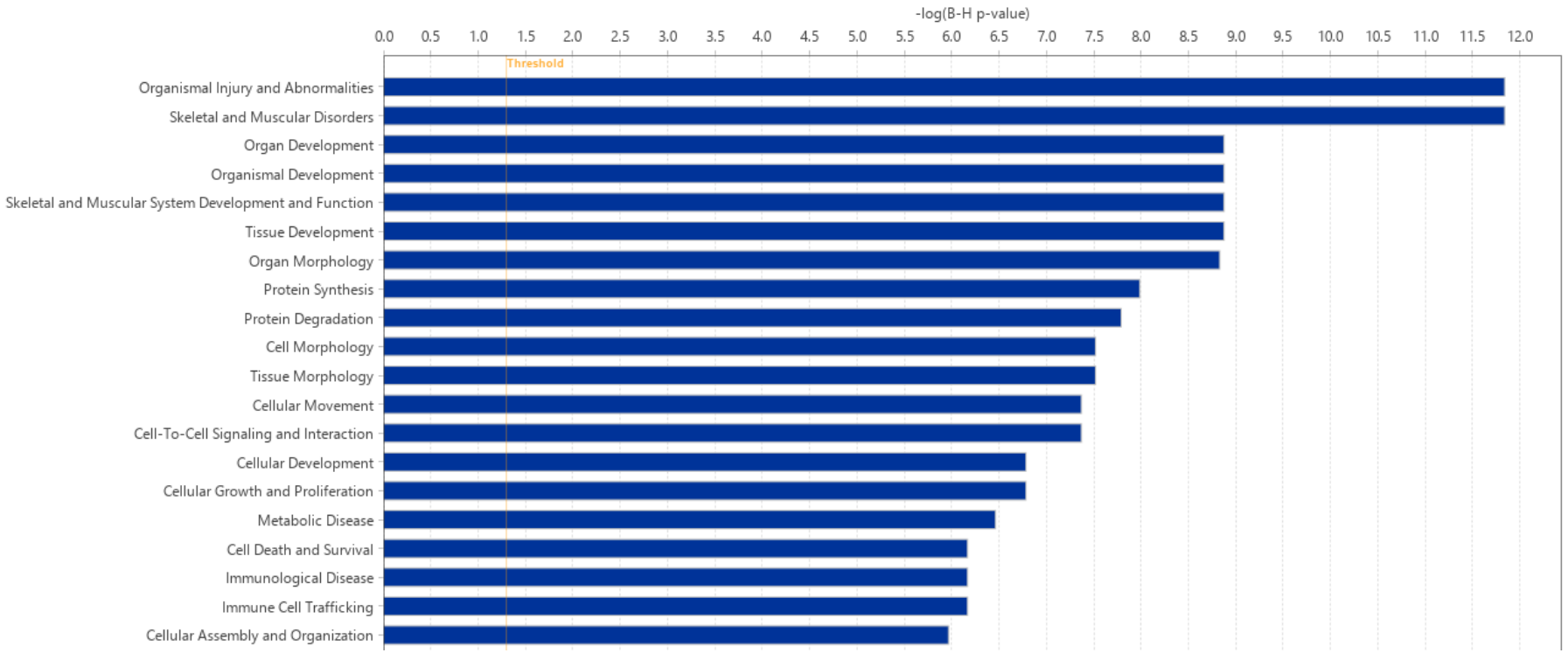
Cellular functions in the fractured hip identified by IPA as being altered from the control frailty CFS 4 group (n=19) in vastus lateralis muscle in patients with CFS 5 during the time of surgery. The x-axis displays the -log of the p value and the y axis displays the cellular functions most affected by the fracture. The p-value associated with each cellular function is a measurement of the likelihood that the association between a set of focus transcripts and a given function is due to random chance. The -log of the p value was calculated by Fisher's exact test right tailed ($p<0.05$). The threshold line corresponds to a p value of 0.05.

Figure 6.10 Top 20 cellular functions identified by IPA in the fractured hip when comparing CFS 6 with CFS 4.



Cellular functions in the fractured hip identified by IPA as being altered from the control frailty CFS 4 group (n=19) in vastus lateralis muscle in patients with CFS 6 during the time of surgery. The x-axis displays the -log of the p value and the y axis displays the cellular functions most affected by the fracture. The p-value associated with each cellular function is a measurement of the likelihood that the association between a set of focus transcripts and a given function is due to random chance. The -log of the p value was calculated by Fisher's exact test right tailed ($p<0.05$). The threshold line corresponds to a p value of 0.05.

Figure 6.11 Top 20 cellular functions identified by IPA in the fractured hip when comparing CFS 7 with CFS 4.



Cellular functions in the fractured hip identified by IPA as being altered from the control frailty CFS 4 group (n=19) in vastus lateralis muscle in patients with CFS 7 during the time of surgery. The x-axis displays the -log of the p value and the y axis displays the cellular functions most affected by the fracture. The p-value associated with each cellular function is a measurement of the likelihood that the association between a set of focus transcripts and a given function is due to random chance. The -log of the p value was calculated by Fisher's exact test right tailed ($p<0.05$). The threshold line corresponds to a p value of 0.05.

Within the fractured hip, two cellular functions are present in all frailty groups when compared with the control CFS. These are organismal injury and abnormality and skeletal and muscle disorders. Schematics for the cellular functions which changed are shown in appendices 9.2.2.5, 9.2.2.6 and 9.2.2.7.

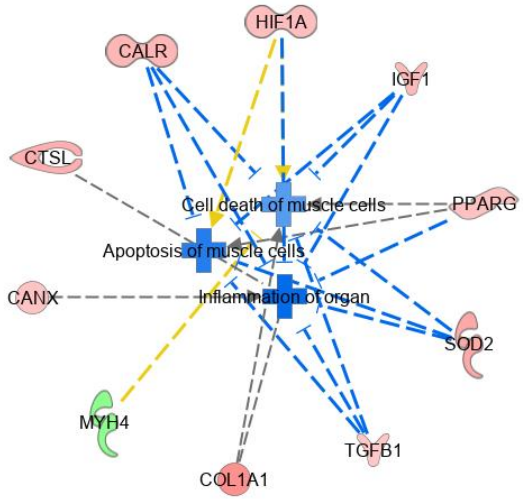
Organismal Injury and Abnormality

In participants with CFS 5, ten mRNAs were identified as altered in abundance relative to the control CFS 4 Figure 6.12A. Nine were up-regulated and one was down-regulated. The most upregulated mRNA from the baseline were COL1A1. The only down-regulated mRNA from the baseline was MYH4. Based upon the collective changes in mRNA abundance from the control, IPA predicted with high confidence, inhibition of inflammation of organ, apoptosis of muscle cells, and cell death of muscle cells.

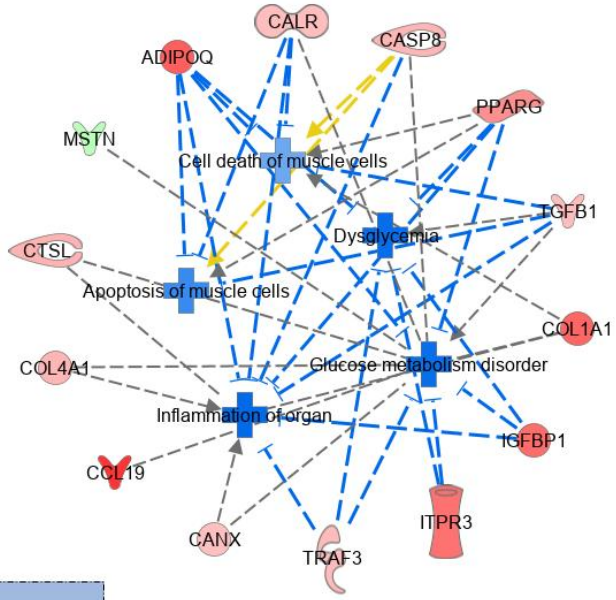
In participants with CFS 6, fourteen mRNAs were identified as altered in abundance relative to the control CFS 4 Figure 6.12B. Thirteen were up-regulated and one was down-regulated. The most up-regulated mRNA from the baseline were CCL-19, ADIPOQ, COL1A1, IGFBP1 and ITPR3. Based upon the collective changes in mRNA abundance from the control, IPA predicted with high confidence, inhibition of inflammation of organ, dysglycaemia, glucose metabolism disorder, apoptosis of muscle cells, and cell death of muscle cells.

In participants with CFS 7, twenty-five mRNAs were identified in as altered in abundance relative to the control CFS 4 Figure 6.12C. Twenty-two were up-regulated and three were down-regulated. The most upregulated mRNA from the baseline were TNC, CCL-19, and SOD2. The most down-regulated mRNA from the baseline were MSTN, and ATP2A1. Based upon the collective changes in mRNA abundance from the control, IPA predicted with high confidence inhibition of inflammation of organ.

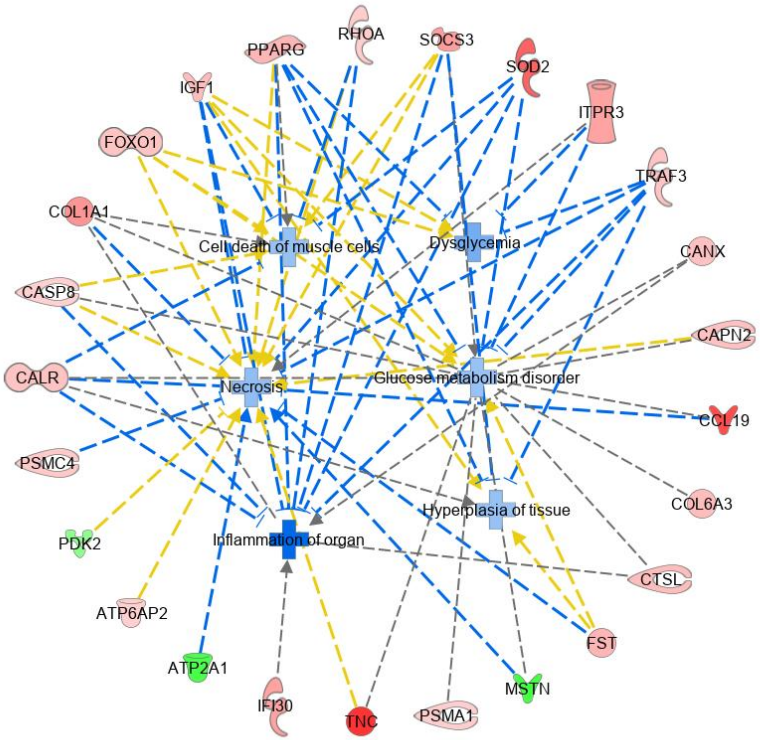
Figure 6.12 Fractured hip – Organismal injury and abnormalities



A. CFS 5

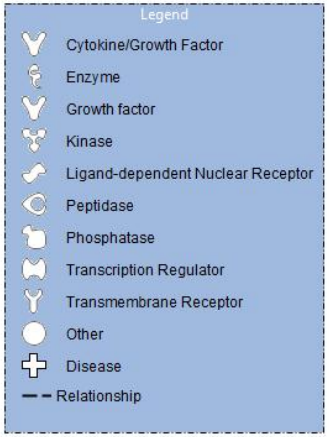


B.CFS 6



C. CFS 7





Differentially regulated mRNAs (outer ring) associated with organismal injury and abnormalities in CFS 5, 6, and 7 compared to CFS 4, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

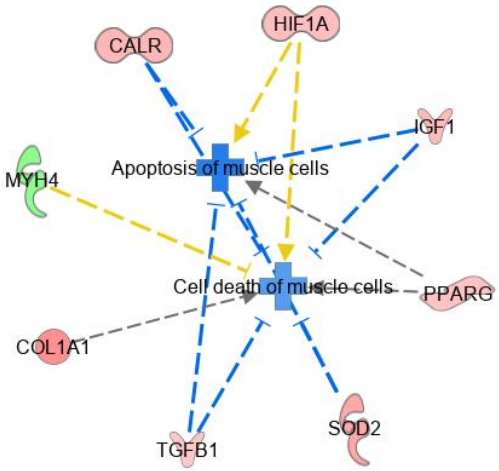
Skeletal Muscle Disorder

In participants with CFS 5, eight mRNAs were identified as altered in abundance relative to the control CFS 4 Figure 6.13A. Seven were up-regulated and on was down-regulated. The most up-regulated mRNA from the baseline were COL1A1. The only down-regulated mRNA from the baseline was MYH4. Based upon the collective changes in mRNA abundance from the control, IPA predicted with high confidence, inhibition of apoptosis of muscle cells, and cell death of muscle cells.

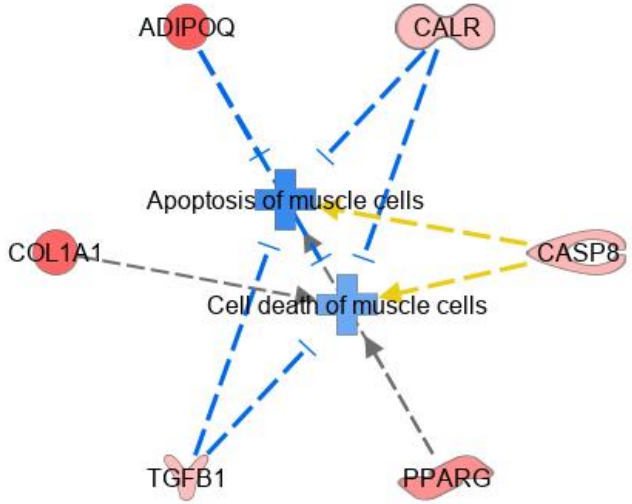
In participants with CFS 6 six mRNAs were identified as altered in abundance relative to the control CFS 4 all of which were up-regulated Figure 6.13B. The most up-regulated mRNA from the baseline were ADIPOQ, COL1A1, and PPARG. Based upon the collective changes in mRNA abundance from the control, IPA predicted with high confidence, inhibition of apoptosis of muscle cells, and cell death of muscle cells.

In participants with CFS 7, fifteen mRNAs were identified as altered in abundance relative to the control CFS 4 Figure 6.13C. Fourteen were up-regulated and one was down-regulated. The most up-regulated mRNA from the baseline were TNC, CCL-19, and SOD2. The most down-regulated mRNA from the baseline were MSTN. Based upon the collective changes in mRNA abundance from the control, IPA predicted with high confidence inhibition of inflammation of organ, and abnormality of lower limb.

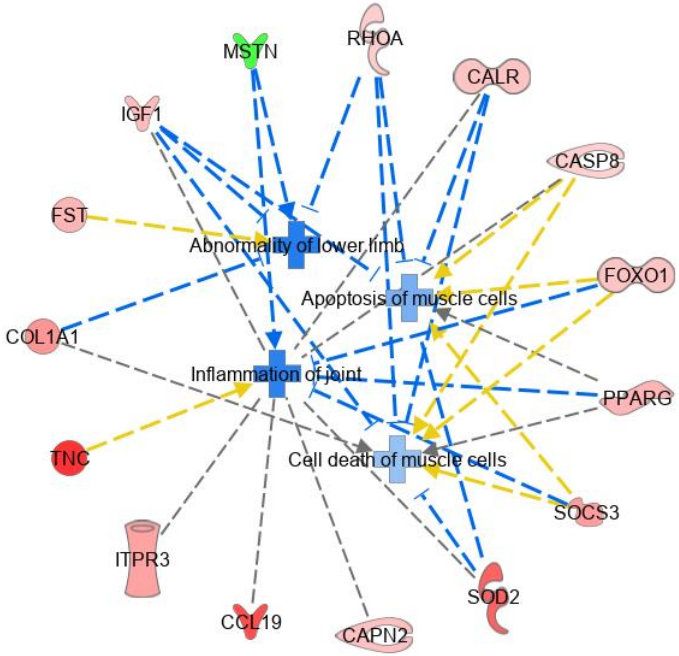
Figure 6.13 Fractured hip – Skeletal and muscular disorder



A. CFS 5

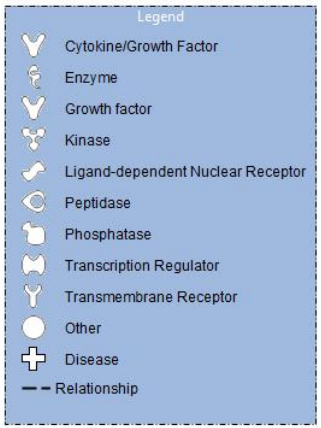


B.CFS6



C. CFS7





Differentially regulated mRNAs (outer ring) associated with skeletal and muscular disorder in CFS 5,6 and 7, compared to CFS 4, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Protein synthesis

In participants with CFS 6 eleven mRNAs were identified as altered in abundance relative to the control CFS 4 Figure 6.14A. Ten were up-regulated and one was down-regulated. The most up-regulated mRNA from the baseline was CCL19. Based upon the collective changes in mRNA abundance from the control, IPA predicted with medium confidence activation of metabolism of protein and synthesis of protein.

In participants with CFS 7 seventeen mRNAs were identified as altered in abundance relative to the control CFS 4 Figure 6.14B. Sixteen were up-regulated and one was down-regulated. The most up-regulated mRNA from the baseline were CCL19 and SOD2 and the most down-regulated was MSTN. Based upon the collective changes in mRNA abundance from the control, IPA predicted with strong confidence activation of metabolism of protein and catabolism of protein.

A summary of the cellular functions within the fractured limb predicted to be activated or inhibited by IPA are shown in Table 6.2

Table 6.2: Summary of cellular functions in the non-fractured and fractured leg. When comparing frailty scores with CFS 4 as the control. Non-fractured leg CFS 4 and CFS 5, CFS 4 and CFS 6 not shown as IPA unable to make predictions based on insufficient number of differentially expressed genes detected. (-) inhibition of function (+) activation of function number of symbols relates to the intensity of confidence of prediction by IPA.

	Non-fractured leg	**Fractured leg**		
	CFS 4 and CFS 7	CFS 4 and CFS 5	CFS 4 and CFS 6	CFS 4 and CFS 7
Organismal injury and abnormality	(---)Organismal death (+) Glucose metabolism disorder	(---) Inflammation of organ (---) Apoptosis of muscle cells (--) Cell death of muscle cells	(---) Inflammation of organ (--) Apoptosis of muscle cells (--) Cell death of muscle cells (---) Dysglycaemia (---) Glucose metabolism disorder	(---) Inflammation of organ (-) Necrosis (-) Cell death of muscle cells (-) Dysglycaemia (-) Glucose metabolism disorder (-) Hyperplpasia of tissue
Skeletal muscle disorders	No prediction	(---) Apoptosis of muscle cells (--) Cell death of muscle cells	(---) Apoptosis of muscle cells (--) Cell death of muscle cells	(---) Inflammation of joint (---) Abnormality of lower limb (-) Apoptosis of muscle cells (-) Cell death of muscle cells

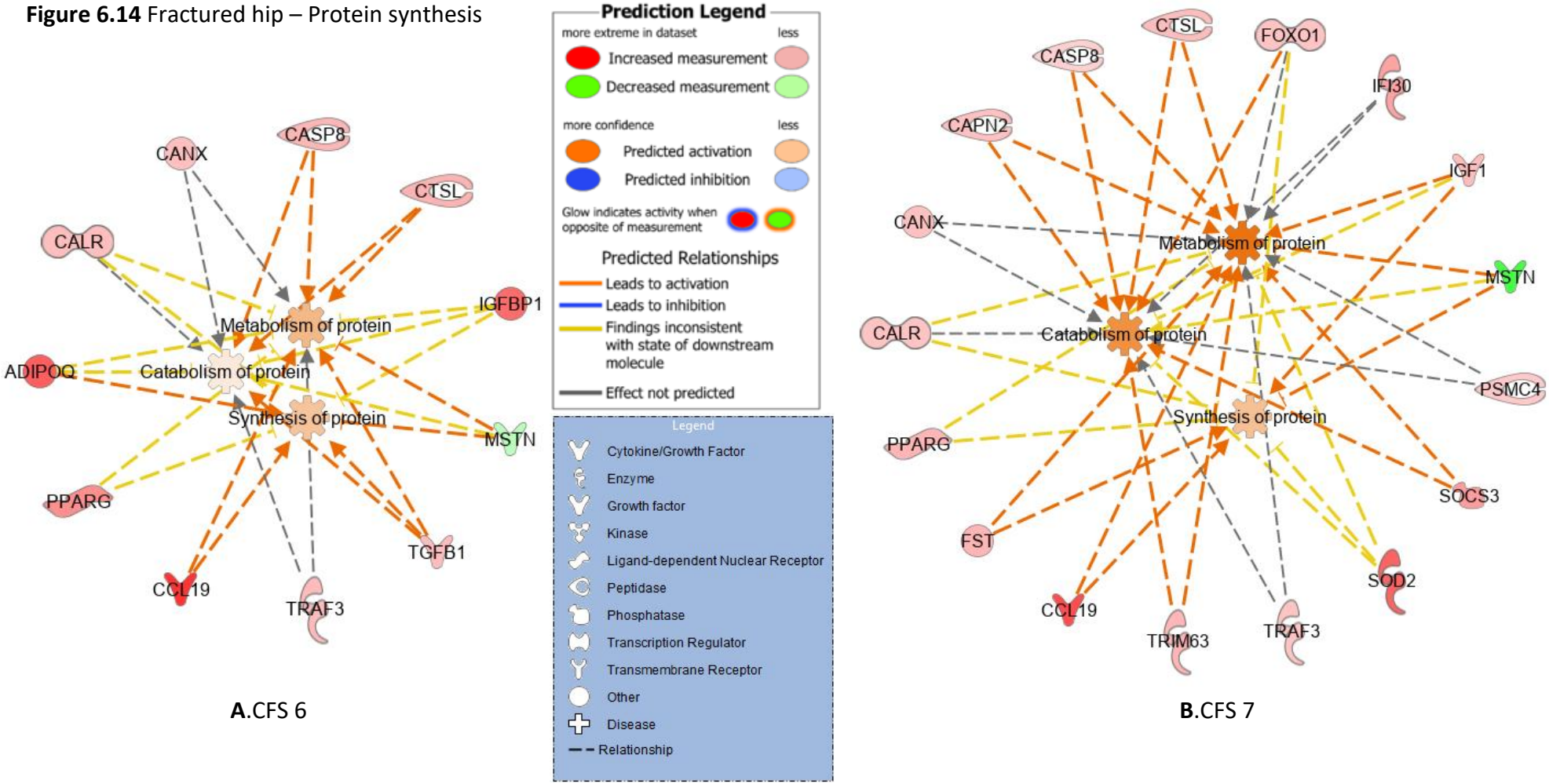
	Non-fractured leg	**Fractured leg**		
Cell death and survival	No prediction	(---) Apoptosis of muscle cells (--) Cell death of muscle cells	No prediction	No prediction
Cellular movement	No prediction	No prediction	No prediction	No prediction
Cellular growth and proliferation	No prediction	No prediction	No prediction	No prediction
Metabolic disease	No prediction	No prediction	(---) Dysglycaemia (---) Glucose metabolism disorder	No prediction
Inflammatory response	No prediction	No prediction	(---) Inflammation of organ (---) Inflammation of absolute anatomical region	No prediction

	Non-fractured leg	**Fractured leg**		
Skeletal and muscular system development	No prediction	(+++) Formation of muscle cells (++) Formation of muscle (++) Quantity of striated muscle (++) Quantity of muscle cells	No prediction	(+++) Formation of muscle cells (+++) Differentiation of myoblasts (+++) Differentiation of muscle cell lines (++) Quantity of striated muscle (++) Proliferation of muscle cells (++) Muscle contraction (+) Differentiation of muscle cells (+) Quantity of muscle cells (---) Contractility of muscle (--) Contractility of skeletal muscle
Cell signalling	No prediction	No prediction	No prediction	No prediction

	Non-fractured leg	**Fractured leg**		
Cellular development	No prediction	(+++) Formation of muscle cells (+) Proliferation of muscle cells (+) Differentiation of muscle cell lines	No prediction No prediction	No prediction
Cellular function and maintenance	No prediction	No prediction	No prediction	No prediction
Tissue development	No prediction	(+++) Formation of muscle cells (++) Formation of muscle (+) Proliferation of muscle cells	No prediction	(+++) Formation of muscle (+) Differentiation of muscle cells
Organ development	No prediction	No prediction	No prediction	(+++) Formation of muscle (++) Proliferation of muscle cells
Small molecule biochemistry		No prediction	No prediction	No prediction
Molecular transport		No prediction	No prediction	No prediction

	Non-fractured leg	**Fractured leg**		
Carbohydrate metabolism		No prediction	No prediction	No prediction
Cell morphology	No prediction	(+) Autophagy	No prediction	(+++) Polarization of cells (+) Transmembrance potential of mitochondria
Organ morphology	No prediction	(++) Quantity of muscle cells	No prediction	(+) Quantity of muscle cells
Tissue morphology	No prediction	(+++) Quantity of cells (++) Quantity of muscle cells	No prediction	No prediction
Protein synthesis	No prediction	No prediction	(+) Metabolism of protein (+) Catabolism of protein (+) Synthesis of protein	(+++) Metabolism of protein (++) Catabolism of protein (+) Synthesis of protein
Protein degredation	No prediction	No prediction	(+) Catabolism of protein	(++) Catabolism of protein
Organismal development	No prediction	No prediction	No prediction	(+++) Formation of muscle

Figure 6.14 Fractured hip – Protein synthesis



A.CFS 6

B.CFS 7

Differentially regulated mRNAs (outer ring) associated with protein synthesis in CFS6 and 7, compared to CFS 4, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

6.6 Discussion

6.6.1 Introduction

This is the first study to be able to compare gene expression in the Vastus Lateralis muscle in both the uninjured and injured hip with samples taken at the time of surgery. Additionally, it is the first study investigating the gene expression in the vastus lateralis muscle of a range of frail patients, who have sustained a hip fracture. The data from this study demonstrates three major findings. Firstly, in all patients, when comparing the fractured hip with the control hip, there are clear differences in gene expression and IPA generated gene networks. This highlights a significant difference in cellular processes which occur in the vastus lateralis muscle in the fractured hip at the time of surgery. Secondly, in the control hip, when stratifying for frailty, there was only a small number of differentially expressed genes, and as a result IPA was unable to identify and make predictions of altered events across frailty across frailty groups. This shows within the control hip; the frailty state is not reflected by differences in gene expression. Lastly, and most importantly in the fractured hip, those in a higher frailty group demonstrate a greater number of differentially expressed genes when compared with those in a lower frailty group. Most cellular processes show a gradation in gene expression and networks associated with frailty, and therefore gene expression and gene networks can be used to classify frailty status in the injured hip. The remainder of the discussion will focus on these major findings.

6.6.2 Vastus lateralis muscle gene expression in the fractured hip versus the control hip.

As far as I am aware no published evidence is available that has considered gene expression in the vastus lateralis muscle in both the non-fractured and fractured hip within the same participant. This comparison is a major advantage of this study and provides a unique insight into the differences in gene expression and cellular functions occurring in both vastus lateralis muscles at the time of hip fracture surgery. The top three most changed cellular functions were cell death and survival, organismal injury and abnormality, and skeletal muscle disorders. Although the top three cellular

functions have been discussed in this thesis for brevity most cellular functions have shown to be significantly different between the limbs and are shown in appendix - 9.2.2. This demonstrates that there is a significant local response in the damaged limb.

Within the top three cellular functions changed, the cellular events which were predicted to be most activated were cell viability and cell viability of connective tissue. The cellular events most predicted to be inhibited were apoptosis of muscle cells, cell death of muscle cells and cell death of connective tissue. The most upregulated genes in the networks were MT1A, SOCS3, TNC, and CCL19. This perhaps suggests that the muscle in the injured leg is trying to prevent apoptosis and cell death and increase its own chance of survival by promoting viability. The breakdown of cellular functions with respect to frailty is discussed further in this chapter.

There is a severe paucity in studies investigating muscle gene expression in patients who have sustained a hip fracture. During the time this study was undertaken some studies have come to light. In these studies patients have been classified according to definitions of sarcopenia a physical component of frailty. One study has shown the expression of 11-beta-hydroxysteroid dehydrogenase type 1(HSD11B1) a major regulator of skeletal muscle function in glucocorticoid induced muscle atrophy and aging. It has been negatively correlated with muscle mass using EWGSOP2 definitions of sarcopenia. But this study was limited due to its sample size of thirty-three patients, did not compare the fractured with the non-fractured hip and did not assess frailty. According to the EWGSOP2 guidelines for sarcopenia muscle strength or performance is the main determining factor for the diagnosis of sarcopenia and low muscle mass informs whether a patient has severe sarcopenia. Therefore, correlations between the gene expressed and muscle strength and performance, rather than mass should have been made.(332)

Kang et al in 2021 reported RNA sequencing profile in patients with and without sarcopenia using AWGS definitions who had sustained an osteoporotic hip fracture. This study included a total of twenty-two samples and found fifteen differentially expressed genes (RUNX1, NGFR, CH3LI, BCL3, PLA2G2A, MYBPH, TEP1, SEMA6B,

CSPG4, ACSL5, SLC25A3, NDUFB5, CYC1, ACAT1, and TCAP) in gluteus maximus muscle biopsies taken at the time of surgery. The cellular functions identified were muscle differentiation capacity and inflammation related genes, skeletal muscle fibre, axon and adipose tissue related genes, mitochondrial energy metabolism related genes.(331) However, this study was again limited by participant number and did not classify participants according to frailty. A strength of this study included the use of Kyoto encyclopaedia of genes and genomes (KEGG) pathway analysis, which is similar to the IPA used in this study. Even though patients were classified according to sarcopenia definitions using AWGS cut-off criteria this would not be relevant for the population of the United Kingdom.

Another study has found over expression of Clusterin in degenerated fibres of vastus lateralis muscle in patients with osteoposoris is closely correlated with IL-6. Isolated Ex-vivo studies on human myoblasts confirmed over expression of clusterin in patient with osteoporosis when compared with patients with osteoarthritis. Treatment with clusterin of isolated myoblasts showed modulation and proliferation, morphological changes, and induction of myogenin (a transcription factor for the development of functional skeletal muscle). This study despite being conducted on patients with hip fracture was limited to forty women. Half of these patients had a hip fracture and half had osteoarthritis. Patients who undergo arthroplasty for a hip fracture are usually frailer than those undergoing the same procedure for osteoarthritis. (338)Patients were not classified according to frailty, and muscle samples were only taken from the fractured hip. The muscle samples were also silenced for the Clusterin gene. Analysis is therefore difficult to compare with the study from this cohort.(339)

A pilot study conducted by McKenzie et al in patients four months following hip fracture surgery, found exercise training to increase the cross-sectional area and strength of the quadriceps muscle and MYD88, TAK1, NFKB1, IL6, SPT2, and CERS1 gene expression decreased after training. MYD88 was inversely correlated with quadriceps cross sectional area. They suggested exercises alters skeletal muscle inflammation and ceramide metabolism associated with toll like receptor signalling in older adults. As this was a pilot study it consisted only of 7 patients, frailty was not

assessed, and gene analysis was undertaken 4 months after hip fracture surgery. It has been proven that there is a significant mortality in those patients on the higher end of frailty within the first 30 days and therefore the recruitment of patients would be biased to those who are potentially fitter and less frail.(340)

The difference in gene expression could be explained by the local inflammatory response of sustaining a hip fracture. There is an immediate response to injury caused by activation of the inflammatory cascade. In muscle this leads to the upregulation of genes encoding inflammatory cytokines and chemokines. There is also activation of the stress response which causes the release of stress responsive genes which may be involved in cellular repair, adaptation to stress and metabolic regulation. This will be explored further in Chapter 6 proteomic analysis.

It could be argued that on the non-fractured side a biopsy was taken through a 5-7mm wound whereas the biopsy from the fractured side was taken through a wound approximately 15cm. the additional insult of surgery may be a confounding factor to inflammation. Inflammation following muscle injury peaks within the first 24-72 hours. Best practice tariff in hip fracture surgery aims for patients with a fragility hip fracture to undergo surgery within 36hours of admission. Inflammation may play a role either through trauma or concurrent systemic illness which precipitated the initial fall. An advantage of this study was that the biopsy was taken relatively simultaneously in both limbs at the time of surgery. This therefore would negate the effect of surgery or systemic inflammation on the muscle sample and difference in time taken. Another factor to consider is the location of the biopsy, in the non-fractured leg it was taken mid-vastus, whereas in the fractured leg it was taken at the proximal aspect of the vastus lateralis muscle. Bosman et al showed similar gene expression is observed in vastus lateralis if muscle biopsies are completed in an hour and irrespective of the leg it was taken from therefore demonstrating one vastus lateralis muscle sample per participant is sufficient to establish a baseline for comparing gene expression.(341) Studies have shown the location of muscle biopsy in vastus laterals has been associated with variability of muscle fibre type and size

characteristics at multiple sites in healthy active individuals, but myonuclear content less variable.(342)

There may have also been a difference in the subtype of hip fracture causing a differential response. Intracapsular hip fractures are contained within the hip whereas extracapsular hip fractures occur distal to the capsule, the local effect of trauma to the muscle may be affected more in extracapsular hip fractures. This was outside the scope of this thesis but could be an avenue for further investigation or analysis. Medical comorbidities may have played a role in inducing a response to trauma, but this would have been a systemic response and may have been seen in both legs but as we go on to see in this discussion there was no difference in frailty states in the gene expression of the vastus lateralis in the non-fractured hip.

Hip fracture can significantly impact muscle gene expression. There is an immediate response to injury caused by activation of the inflammatory cascade. In muscle this leads to the upregulation of genes encoding inflammatory cytokines and chemokines. There is also activation of the stress response which causes the release of stress responsive genes which may be involved in cellular repair, adaptation to stress and metabolic regulation. Muscle adaptation and remodelling occurs due to changes in myogenic regulatory factors and extracellular matrix remodelling. These processes are essential for muscle regeneration and repair, tissue repair and scar formation. Prolonged immobilisation following hip fracture can lead to muscle wasting and atrophy and the activation of genes coding for this. Genes associated with muscle protein breakdown may also be upregulated as well as alterations in the expression of genes encoding for different muscle fibre types reflecting adaptations to activity and disease. It is therefore key to understand how frailty is related to the cellular processes of the non-fractured and fractured limb. This information can then be used as potential targets for further investigation and research for treatment to improve patient outcomes following this devastating injury.

6.6.3 Vastus lateralis muscle gene expression in the control hip comparing frailty states.

In the control hip, as frailty increases, there's a rise in the number of differentially expressed genes. However, due to the limited gene count, IPA's predictive capacity is constrained, especially when comparing moderately (CFS 6) and mildly (CFS 5) frail groups with the control group (CFS 4). Predictions were restricted to molecular transport, organismal injury, carbohydrate metabolism, and small molecule biochemistry. Cellular processes primarily activated involve D-glucose concentration and carbohydrate metabolism, with some indication of inhibition of organismal death and lipid concentration. Despite statistical significance, the gene networks are simplistic, rendering differentiation of frailty states based on gene expression in the non-fractured hip unfeasible.

Studies investigating gene expression have been completed in patients without hip fracture. Andreux et al have shown mitochondrial function to be impaired in the skeletal muscle of the pre-frail elderly. However, despite careful selection of a pre-frail population a comparison between frail patients was not made. Comparison to an aged matched physically robust cohort was completed. Their study was limited in the number of patients with twenty-one recruited overall. Frieds frailty phenotype was also used to classify frailty in these patients, which does not take into the cognitive impairment factor and reduces the assessment of frailty to physical deficiencies. None of the patients analysed in Andreux et al study was as frail as the patients discussed in this thesis.(343)

Drummond et al showed physical inactivity in frail older women is associated with a down regulation of ubiquitin-mediated and autophagic lysosomal skeletal muscle gene expression.(344) However, only fourteen patients were recruited to this study and patients were divided into active and inactive groups based upon performance, self-reported assessments and not frailty. Gene expression analysis was undertaken using western blotting which may not be as reliable as previously assumed. A primary antibody against the protein of interest must be available and therefore this technique

is limited in the detection of proteins. Many antibodies exhibit off target effects by interacting with other proteins and the technical demand on the scientist is paramount and proper experimental techniques and strategies must be employed.(345)

De Jong et al found the top ranked pathways differ between males and females but were present and altered in the same direction in both sexes. The top ranked differentially expressed processes in males was oxidative phosphorylation and in women was cell growth mediated by AKT signalling. These processes were associated with skeletal muscle aging, but differential expression is sex specific. This study had a slightly higher number of patients included (fifty-four) and used a similar technique as described in this thesis with the use of IPA but these patients did not sustain a hip fracture. Frailty was classified according to Fried frailty score, but analysis was not stratified for frailty, comparisons were completed for sex only.(346)

This study is clearly different from the existing literature for three clear reasons. The first is having clearly classified participants into different frailty subgroups based upon the Rockwood classification, which includes pre-frail, mild, moderate, and severe frailty. Secondly it investigates participants who have sustained a significant trauma in the form of a hip fracture. Lastly it has undertaken an analysis in the control non-fractured hip. Comparisons and prediction of gene expression, networks and cellular processes have been undertaken using real-time qPCR and IPA analysis.

This study has clearly demonstrated when stratifying for frailty there does not appear to be a difference in gene expression in the vastus lateralis muscle of the non-fractured hip. Therefore, using gene expression to identify frailty cannot be completed in the non-broken hip. However, with the initial findings of differential gene expression between the non-broken and broken hip this again demonstrates that the gene expression and networks shown are likely to be due to a local rather than a systemic effect.

6.6.4 Vastus lateralis muscle gene expression in the fractured hip comparing frailty states.

In the fractured hip the degree of differentially expressed genes increased with increasing frailty. Some cellular functions showed a graded expression with increasing frailty. The functions which showed a graded expression are discussed here.

Organismal injury and abnormality

The cellular function of organismal injury and abnormality was in the top three most significant changed functions when comparing each CFS score 5-7 with the control CFS 4. As the frailty increased from mild, to moderate and severe the complexity of the gene network and number of differentially expressed genes within the network also increased. Inhibition of inflammation of the organ was commonly predicted by IPA with high confidence across all frailty groups. However, the confidence of prediction of inhibition of cell death of muscle cells decreased as the frailty scale score increased. This was also true of apoptosis of muscle however the prediction of inhibition was not present in CFS7.

Skeletal and muscle disorders

The cellular function of skeletal muscle disorders was in the top three most significant changed functions for CFS 5 and CFS 7, but not in CFS 6 however there were enough differentially expressed genes for IPA to make predictions in function across all frailty groups. Apoptosis of muscle cell and cell death of muscle cells were predicted to be inhibited, this prediction of inhibition decreased as the frailty score increased, for both these functions.

These cellular functions demonstrate that despite the intensity of prediction of cellular function increasing as the frailty score increases, the net effect is the cells lose their ability to prevent cell death of muscle cells and apoptosis. As a person becomes frailer it appears the muscle is working harder to protect itself, but the final function is potentially not happening or to a reduced extent. This process would fit with the

clinical picture of frailty whereby the frailer a person is the more their ability to bounce back from injury or illness becomes impaired.

Skeletal muscle has unique features as it is multinucleaeted and can undergo individual myonuclear apoptosis as well as complete cell death. Preliminary studies have shown skeletal muscle apoptosis in age related muscle loss with many studies conducted in animals. Only three reports on skeletal muscle apoptosis with aging in humans are available but none have investigated the specific biochemical pathways of apoptosis or the functional implications.(347-349) Whitman et al found a significant increase of apoptotic myonuclei in the vastus lateralis of old subjects compared to young controls.(349) Apoptosis is believed to be the mechanism to represent the final common pathway through which physical frailty ensues.(350) It has been correlated with reduced walking speed and muscle volume.(351) The contribution of apoptosis to sarcopenia is strongly supported by the observation that both the activation and severity of atrophy is greater in muscles with fast twitch fibre dominance relative to slow twitch fibre (350) which is the case in vastus lateralis.(352) behavioural, pharmacological or genetic interventions have been proven to be effective at rescuing muscle mass and function in old age and mitigate the severity of apoptosis.(353)

The cellular function of protein synthesis was not predicted by IPA when comparing CFS 5 with 4, however it was present when comparing CFS 6 and 7 with CFS 4. The complexity of the gene network and prediction of function increased with increasing frailty. This was evident with the increase in the prediction of activation of metabolism of protein and catabolism of protein. The intensity of prediction of synthesis of protein did not change between frailty states.

Observing the rosettes of networks predicted by IPA which have been described in this chapter an abundance of TNC, CCL19, and SOD2 and depletion of MSTN were main genes highly predicted by IPA when observing the above predicted cellular functions. These genes increased or decreased in their predicted expression as the frailty increased.

TNC plays a role in the patterning and differentiation of muscle precursor cells, influencing muscle fibre formation and alignment. Following muscle injury TNC expression is upregulated. It contributes to the formation of the provisional extracellular matrix scaffold at the injury site, serving as a substrate for migrating and proliferating myogenic precursor cells (satellite cells). It also facilitates muscle regeneration and repair. ln both healthy and diseased skeletal muscle, TNC is involved in the remodelling of the extracellular matrix. It does this through interactions with other matrix components, such as fibronectin and collagen, and with cell surface receptors, including integrins, to modulate cell adhesion, migration, and signalling processes. TNC increases both pro-and anti-inflammatory signalling pathways and has been identified as an essential factor in tissue injuries such as damaged skeletal muscle, heart disease, and kidney fibrosis.(354)

CCL19, is involved in inflammatory processes which can influence skeletal muscle health and function. While CCL19 role in immune cell trafficking primarily occurs within lymphoid tissues, systemic inflammation can affect skeletal muscle through circulating cytokines and immune cells. Chronic inflammation, characterized by dysregulated cytokine production and immune cell infiltration, has been implicated in muscle wasting conditions such as sarcopenia. Immune cells play roles in muscle repair and regeneration after injury. While CCL19 direct involvement in this process is not well-defined, alterations in immune cell recruitment and function mediated by CCL19 could indirectly influence muscle repair or contractility.

Skeletal muscle is a highly metabolic tissue that produces reactive oxygen species as by-products of energy metabolism, particularly during periods of exercise. Excessive reactive oxygen species can cause oxidative damage to muscle cells, leading to impaired contractile function, inflammation, and muscle fatigue. SOD2 helps mitigate this oxidative stress by converting superoxide radicals (O^{2-}) into less harmful hydrogen peroxide (H^2O^2) within the mitochondria, thereby protecting muscle cells from oxidative damage. By detoxifying superoxide radicals, SOD2 helps maintain mitochondrial function and integrity, preserving energy production and preventing mitochondrial dysfunction-induced muscle damage.(355)

Skeletal muscle undergoes age-related changes, including a decline in antioxidant defences and an increase in oxidative stress. Reduced SOD2 activity and increased oxidative damage have been observed in aging skeletal muscle, contributing to age-related muscle dysfunction, weakness, and sarcopenia.(356) Strategies aimed at enhancing SOD2 activity or expression may help mitigate age-related muscle decline and preserve muscle function in older adults. SOD2 plays a critical role in protecting skeletal muscle from oxidative stress and maintaining muscle health and function. Understanding the mechanisms underlying SOD2-mediated antioxidant defence in skeletal muscle may lead to the development of novel strategies for preventing muscle damage and promoting muscle adaptation and performance.(355)

MSTN is a negative regulator of muscle growth. It supresses muscle hypertrophy and limits muscle mass. Reduced muscle mass and strength are known risk factors for falls and fractures. The MSTN gene codes for myostatin a myokine. It is primarily produced in skeletal muscle and acts locally to limit muscle growth and size by inhibiting the proliferation and differentiation of muscle precursor cells (myoblasts) and promotes muscle protein degradation.(357) Mutations in the MSTN gene have been observed in various animal species and are associated with a doubling-muscle phenotype. Myostatin has garnered significant interest as a potential therapeutic target for treating muscle wasting conditions and promoting muscle growth in various clinical settings, including sarcopenia (age-related muscle loss), cachexia (muscle wasting associated with chronic diseases such as cancer), and muscular dystrophies. Strategies aimed at blocking myostatin signalling, such as myostatin inhibitors or gene therapy approaches, are being explored for their potential to enhance muscle mass and function in these conditions.(357)

Existing literature has shown serum MSTN levels to be a potential biomarker in the prediction of frailty in patients with chronic heart failure.(358) Serum myostatin levels have also been highlighted to be a potential biomarker to monitor sarcopenia in hip fracture patients undergoing MDT rehabilitation and nutritional treatment.(359) This is contradictory to what this study has found however another study has shown serum MSTN transiently decreased following hip fracture surgery.(360) The main difference

in this study was MSTN levels were from muscle samples rather than serum, and as previously explained MSTN codes for myostatin which acts locally in skeletal muscle.

Study found higher serum myostatin to be associated with better physical fitness. Leaner body composition, greater upper and lower limb strength, better TUG test performance. They found higher concentration of MSTN in non-frail than in frail patients. Physical exercise increased MSTN concentration in men but not women. Although in this study low level of MSTN was found in higher frailty patients (361)

6.6.5 Reflection and Limitations

This study was undertaken at the time of and height of the Sars-Cov-2 pandemic. Subsequently much of the research capacity including laboratory equipment and personnel previously available was seconded to other fields to further Covid-19 research and to facilitate the production of a vaccine. Research nurses and clinical staff were also reinstated to frontline services to aid in the care of patients. Therefore, for the first 9 months of the research time of this PhD, this study was on hold until the University gained permission to open non-covid related studies. The research team then felt due to the time constraints to limit the muscle tissue analysis undertaken to the gene expression. The histological analysis and intramyocellular lipid content of the muscle tissue which was one of the measures to be determined according to the FEMUR study was not completed. It is important to understand the macro and microstructure of the tissue samples with respect to frailty.(362) Despite key genes being expressed they may translate into proteins with specific functions.

The measurement of gene expression is the evidence that transcription is taking place. It does not signify that translation is occurring. Translation requires the formation of amino acid chains and quaternary structure folding to form proteins, and it is these proteins which cause the resultant cellular effects to occur. This is overcome with proteomic analysis, whereby the concentration of proteins within the substrate are investigated. However, the assessment of gene expression is the first point of call in understanding the cellular pathways and mechanisms involved with a given function.

To understand the underlying mechanisms and protein expression the origin of expression also needs to be investigated. This is undertaken through immunohistochemistry and immunofluorescence. Comparative analysis between the injured and uninjured leg in this study strengthens the data and makes the observations we have seen more likely to be genuine.

Macro and microstructure of tissue samples not analysed with respect to frailty states. Fibre type is also important modification of fibre type may explain clinical differences in performance.(362) Due to the time constrains of the study and the Sars-Cov-2 pandemic the morphological structure of the tissue was not investigated or correlated to clinical frailty. This would have given unique insight as to the quantity and quality of fibre type which would complement the gene expression data.

Multiple factors affect gene expression including recovery and rehabilitation.(363) Gene expression may have changed over the period of recovery. It would have been ideal to undertake repeat muscle samples at 1 week and 90 days to ascertain change in gene expression and if this correlates to frailty state. However, due to the nature of the participants involved in this study having cognitive impairment ethically this would have been difficult to obtain a muscle sample. A general anaesthetic would have increased risk to the participant, and performing the procedure under local anaesthetic would have been technically challenging as the participant may not have remained still long enough or tolerated this. This is irrespective of bringing frail patients back to a secondary care setting where there is an increased risk of acquiring an infection from this setting. Lastly the increased mortality rate of those patients who were frailer would lead to bias in the acquisition of muscle samples from lower frailty groups.

We have shown that CFS 7 do not improve with regards to their subjective and objective measures of muscle function. Is this a result of the injury or the inability to recover from the injury.

7 The association between inflammatory markers at the time of surgery and its relationship with 6-month survival in patients with fragility hip fracture.

7.1 Overview

This chapter evaluates 92 inflammatory markers in pre-operative serum samples from frail patients with hip fracture. This has been achieved through proteomic analysis. Comparison of these markers has also been undertaken non-frail participants with no fracture, non-frail participants with severe orthopaedic injuries, and prefrail patients with no fracture. Following correction for multiple testing, adjustments for comorbidities, and demographics, statistical analysis demonstrated of the markers tested, fibroblast growth factor (FGF-23) and interleukin-15 receptor alpha (IL15RA) were significantly correlated with 6-month mortality.

7.2 Introduction

According to the World Health Organisation hip fractures permanently disable 50% of sufferers and have an approximate 24%, 1-year mortality.(364-366) The mortality rate after hip fracture is significantly higher compared to the age and gender-matched population.(367) Despite comprehensive best practice surgery and rehabilitation, post-surgical mortality has been static over the past decade.(368-371) Patients with a hip fracture are at substantial risk of major complications which include cardiovascular, infectious, neurocognitive and mortality.(372) Several systematic reviews have consistently found most of the associated factors for functional recovery and mortality of elderly hip fracture are biological, sociodemographic, or inherent to patients' baseline characteristics.(373-375)

Frailty is defined as a status of extreme vulnerability to stressors, (as discussed in Chapter 1.4) is highly correlated with higher systemic inflammation.(376) Importantly, the main causes of 12-month mortality for elderly hip fracture patients are

cardiovascular disease and Alzheimer's disease, (377) both of which have inflammation as a central mechanism.(378, 379)

The specific mechanisms mediating the increased risk of mortality for these patients is unclear. Increased risk of mortality is associated with inflammation,(18, 19) if there is an identifiable mechanism then there is the potential for an intervention point. The responsiveness of such mechanisms to surgery, and rehabilitation are crucial to understanding potential therapeutic interventions to improve outcomes. the development of interventions that improve morbidity and mortality outcomes following fragility hip fracture surgery. We have hypothesized that higher rates of mortality among individuals with pre-operative frailty might be driven by specific inflammatory pathways and modulated by delays to theatre. To investigate this, we used a cross-sectional study design and proteomic analysis in an attempt to identify relevant pathways.

7.2.1 Protein expression and proteomic analysis

The proteome is the total protein content of a cell, as defined by its localization, interactions, post-translational modifications, and turnover at a given time. The action of proteins produced by cells within the body regulates cellular and organ function. This is achieved through a complex process of transcription of deoxyribonucleic acid (DNA) into messenger ribonucleic acid (mRNA) and translation of mRNA into a polypeptide chain composed of amino acids. DNA is a polymer made up of two polynucleotide chains that form a double helix. It contains the genetic information necessary for the growth, development, and reproduction of all living organisms. In the cell nucleus, DNA is transcribed into messenger RNA (mRNA). mRNA is a single-stranded molecule that corresponds to a gene's genetic sequence. It leaves the nucleus and is converted into protein. A protein is a large biomolecule composed of one or more long amino acid chains. mRNA is a measure of which genes are activated in a cell, but it can be degraded, cycle back on itself, or not be translated, so while it is a measure of all the genes that are activated, it is not an exact measure of all the proteins that will be expressed. The singular strand of mRNA is translated into a

polypeptide chain consisting of amino acids, with each of the mRNA's three bases coding for a specific amino acid.(380)

The primary protein structure of the polypeptide chain locally folds into the secondary protein structure. It creates either a α-helix or a β-pleated sheet. On the same polypeptide chain, different local folding can occur. The secondary structure then transforms into the tertiary structure, which is a three-dimensional folding that creates bonds between sheets and helices. Finally, a quaternary structure is formed, typically involving more than one polypeptide chain, and in this form, the molecule can act within the cell. Haemoglobin, for instance, consists of two alpha units and two beta units composed of more than one amino acid polypeptide chain. Proteins frequently undergo post-translational modifications, such as phosphorylation, to enable the protein to act in specific ways within the cell. This may be to become active, carry out functions within the cell, or even become inactive. Protein structures are considerably more complex than mRNA structures. From DNA to mRNA to protein synthesis, complexity significantly increases. Over a million proteins may be encoded by 22,000 genes even still this may be a conservative estimate. Consequently, quantifying protein concentration is more difficult than measuring DNA or mRNA. (380) Thus selection of a proteomic analysis allows for inference as to the activity levels of specific pathways in response to an external exposure (in this case delay to surgery and frailty)

Proteomics is a method with a high throughput for measuring all of the proteins in a cell or tissue. Traditionally, mass spectrometry has been used to identify and quantify proteomes. The sample is deposited in a mass spectrometer following initial quality control. Here, the sample is transmitted through a source of electron beams. The ions are accelerated towards a magnet, and due to their differing charges, the magnet deflects them in varying proportions. The quantity of deflection is dependent on the mass-to-charge ratio of the sample, which allows the particle to be identified by the detector. The atomic mass or mass:charge ratio is plotted against the relative abundance to create a graph. This produces both the specific protein type and the sample's protein concentration. When protein samples are sent for mass

spectrometry and the facility performs initial quality control. A count table emphasising the number of present proteins is generated. Then, an algorithm is developed to assist in analysing the changes and their significance. For a visual representation of the data, heat maps and volcano diagrams are generated. Once concluded, it is possible to observe specific proteins of interest. In addition to being able to identify and quantify the quantity of a substance in a sample, mass spectrometry can also differentiate between molecules with the same mass but distinct chemical structures. However, mass spectrometry is only effective across larger atomic masses, or very low charge. It cannot distinguish between optical and geometric isomers and cannot differentiate between isomers of molecules with the same mass:charge quotient.(381)

Protein expression mapping and protein interaction mapping are two classifications of proteomics. Protein expression mapping can provide understanding of the post-translational modifications of expressed proteins under various environmental or disease conditions. Mapping protein-protein interactions identifies the interaction partners for each protein encoded by a cell. Plasma is the body fluid of choice for biomarker discovery due to its exceptional accessibility and abundant protein repertoire, which mirrors the pathophysiology of living organisms. Despite advances in proposed workflow combinations of multiple biomarkers and clinical metadata analysis, the dynamic range of plasma proteins can span more than 10 orders of magnitude. This poses significant limitations for mass spectrometry. Preanalytical variability, depth of proteome coverage, and robustness of discovered biomarkers remain mass spectrometry's greatest obstacles. In plasma proteomics, affinity-based proteomic technologies are gaining increasing attention.(382)

Olink Proteomics (Uppsala, Sweden) has devised a novel technology known as the proximity extension assay (PEA). It combines polymerase chain reaction (PCR) in real time with multiplex immunoassays. Dual recognition of a targeted biomarker by a matched pair of antibodies labelled with a specific DNA oligonucleotide is the foundation of PEA. This biomarker-specific DNA barcode is quantified using microfluidic qPCR, which permits high throughput relative quantification of up to 1161

human plasma proteins using a few microliters of biofluid (1µL for the quantification of 92 biomarkers).(383) The use of highly specific antibodies and primers designed for a specific target increases the sensitivity and specificity of assays performed on samples. Multiple internal controls that monitor each stage of the reaction aid in avoiding unspecific events and minimising background noise. The specific protein panels are designed by Olink and aim to include whole pathway analysis to enable a contextual understanding of the proteome for a specific indication (e.g. in this case inflammation). With 14 human panels, each containing 92 biomarkers, and a dynamic range of over 10 orders of magnitude, levels below picograms per millilitre (pg/mL) can be accurately quantified. In recent years, the number of research articles presenting clinical data using PEA technology has increased exponentially, surpassing the annual number of articles presenting mass spectrometry-based approaches.

One study has shown when comparing PEA with mass spectrometry in 173 samples there is small overlap of the detected proteins (35 overlapping proteins).(384) An advantage of mass spectrometry is the ability to detect a range of post translational modifications and large fraction and abundance proteins. However, there is a limit in its ability to detect low abundance low molecular weight fractions of plasma proteome. Olink PEA was able to detect hidden and hard to reach proteome enriched in biological relevant small molecules, such as cytokines, chemokines and interleukins. This high sensitivity to detect low abundance plasma proteins has made it the gold standard in clinical diagnostics. This study recommended the use of both mass spectrometry and PEA to determine absolute quantification and sensitivity of the proteome map.(384)

7.3 Inflammatory markers and hip fracture

This chapter expands on the inflammatory markers in hip fracture that have been discussed previously in Chapter 5 and in the introduction Chapter 1. The addition of proteomics provides complementary information to the RNA based cytokine expression work presented in the previous chapter and gives a greater insight into actual protein activity accounting for post-translational modifications. The

inflammatory burden in patients with fragility hip fracture have been poorly investigated. Studies have shown cytokines (CRP, IL-10, IL-6 and TNF-α) have been shown to play a role in helping to predict complications and mortality.(385) Studies investigating inflammatory markers have only been able to investigate a few different select inflammatory proteins at a time. This study is unique as it uses proteomic analysis to enable the investigation of a wide range of biomarkers.

7.4 Aims and objectives.

7.4.1 Aims

The aim of this study was to analyse the inflammatory proteomes expressed at the time of surgery and their relationship with frailty and mortality.

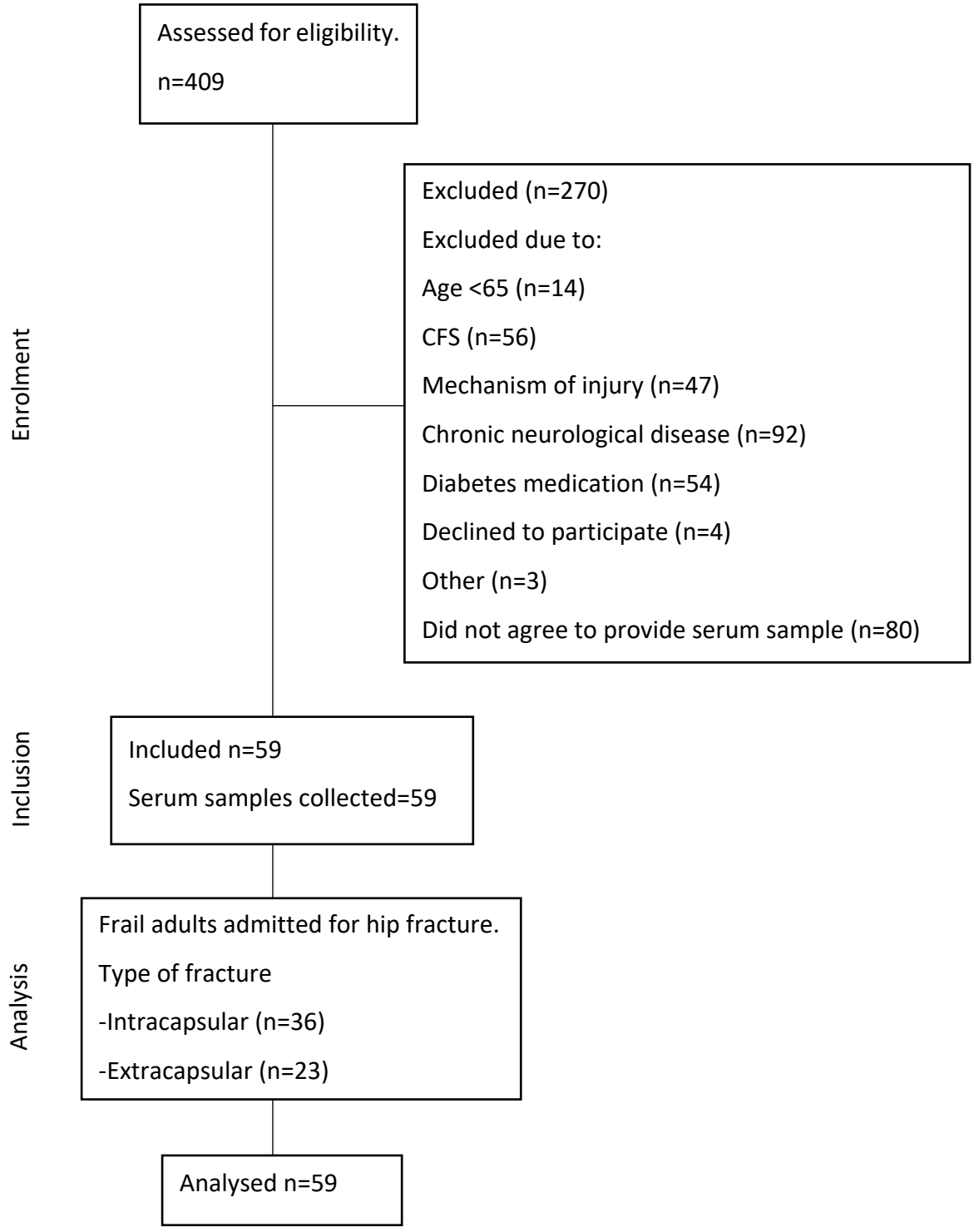
7.4.2 Objectives

Identify the inflammatory proteomes expressed at the time of hip fracture surgery using proteomic analysis. To identify if any inflammatory proteins expressed have a relationship with 6-month mortality.

7.5 Methodology

This was a prospective, observational cohort study, serum preoperative samples were collected from a sub-cohort of frail hip fracture patients, with outcomes assessed at 6-months. Analysis of serum markers aimed to test candidates as predictors of postoperative mortality. Levels of the significantly associated serum markers were also compared to positive and negative controls from additional comparator cohorts. CONSORT flow chart of FEMUR cohort through study is shown in Figure 7.1.

Figure 7.1 CONSORT flowchart of FEMUR cohort through study.



Fifty-nine frail hip fracture patients from the Functioning of Elder Muscle; Understanding Recovery (FEMUR) cohort were consented to give blood samples at induction of anaesthesia (clinicaltrials.gov NCT04764617). This cohort recruited frail individuals admitted to hospital with a hip fracture. All participants were over 65 years of age, with a clinical frailty score of 4 or above and had suffered a hip fracture following a fall. All patients were recruited from Nottingham University Hospitals NHS Trust.

Four comparator cohorts were assembled (Table 7.1), including an established local registry,(76) which includes data collected from the electronic health records (EHR) for all individuals who underwent a hip fracture surgery during the same period at Nottingham University Hospitals Trust. Patients were sequentially admitted neck of femur fracture patients between 01/01/2020 and 31/12/2022.

	Functioning of Elder Muscle Understanding recovery (FEMUR)	PANdemic Tracking of HEalthcare woRkers (PANTHER)	Orif Procedure mEchanisms of Rib Fixation (OPERA)	Molecular Pathways Involved in Knee Pain: an Observational Study
n	59	62	30	12
Description	Frail elderly participants admitted with a hip fracture.	Non frail participants, with no fracture	Non frail participants with severe orthopaedic injuries (Multiple rib fractures)	Prefrail participants with knee arthritis Prefrail No fractures
Reason for inclusion	Cohort of interest and investigation	Healthy control cohort	Positive control of severe orthopaedic trauma in the absence of frailty	Positive control of frailty in the absence of orthopaedic trauma
Mean Age Years (SD)	87.7 (5.9)	42 (11.57)	60.4 (14.2)	70.1 (7.16)
Sex n (%)				
M	9 (15%)	16 (26%)	21 (70%)	3 (26%)
F	50 (85%)	46 (74%)	9 (30%)	9 (74%)

Table 7.1 Descriptive characteristics of four comparator cohorts; hip fracture (FEMUR) cohort, healthy controls (PANTHER), multiple rib fractures (OPERA) cohort, and Molecular Pathways Involved in Knee Pain: an Observational Study.

The PANdemic Tracking of HEalthcare woRkers (PANTHER) cohort involved asymptomatic healthcare workers who were assessed for SARS-CoV-2 infection during the first UK wave of the Covid-19 pandemic using symptom questionnaires and antibody testing. These participants donated blood to the Nottingham Tissue Bank for antibody testing and completed up to 16 weeks of symptom questionnaires. A randomly selected subset of this cohort has been included in this study as the negative control group. Sixty-two participants from the PANTHER cohort were randomly selected to be included in this study as the healthy control group, ensuring ethnic diversity of the sample. This study was initially under a Human Tissue Authority licence in Nottingham (Licence number: 11035) and subsequently received ethical approval from Northwest – Greater Manchester South Research Ethics Committee, reference 20/NW/039 (Registration number: NCT04318314). This group was included as a healthy control cohort.

The Orif Procedure mEchanisms of Rib Fixation (OPERA) cohort is an embedded mechanistic substudy within the ORiF's randomised controlled trial (RCT) aimed at investigating the mortality, quality of life and cost effectiveness of operative rib fixation plus supportive management compared to supportive management only for patients admitted with three or more rib fractures. The OPERA sub-study which collects preoperative sera for these impact fracture patients has been included in this study as the positive control group. This group was included as a positive control of severe orthopaedic trauma in the absence of frailty.

The prefrail sub cohort included a subset of participants over 65 years of age who were recruited into an OA study and donated serum samples. Participants who were over 65 and considered to be prefrail using the FRAIL scale combining the 3 items addressing Fatigue, Resistance (climbing steps), Ambulation (walking), together with weight loss and morbidity counts. Participants fulfilling 1 or 2 criteria may be classified as "pre-frail" and 3 or more as frail. This study received ethical approval from East Midlands Research Ethics Committee, reference 20/EM/0065 (Registration number: NCT0442452). This group was used as a positive control of frailty in the absence of orthopaedic trauma.

In the FEMUR cohort, the primary outcome measure was 6-month postoperative mortality. Recorded data also included the Charlson comorbidities index (CCI), (386) a measure of comorbid condition severity and has been widely validated. (387) The Rockwood Clinical Frailty Scale (CFS) a judgment-based frailty tool that evaluates specific domains including comorbidity, function, and cognition to generate a frailty score ranging from 1 (very fit) to 9 (terminally ill).(388) The Nottingham Hip Fracture Score (NHFS) a validated predictor of 30-day mortality in neck of femur fracture patients using demographic and clinical parameters recorded immediately preoperatively.(76, 77) and lastly the presence of renal disease coded as chronic kidney disease (CKD) in addition to the comorbidities which are recorded as part of the CCI at admission.

Laboratory measurements:

Blood samples were taken and processed according to the standard operative procedure protocol FEMUR study preparation of serum samples V1.1 (appendix 9.3.1) Blood samples were obtained in the anaesthetic room prior to surgery from patients recruited to the FEMUR study. Samples were taken using a butterfly needle and a vacutainer. Blood was stored in a blood collection tube known as serum separating tube because it contains two additives that separate the serum from the blood's cellular components. The two additives are silica (a clot activator) and a separation gel that creates the physical barrier between serum and cells. Once the sample was obtained it was allowed to rest for 30mins for the clot activator to work. Following this the samples were then centrifuged at 3000RPM for 5 mins. The serum was aliquoted using a micropipette and stored into labelled cryovials and frozen at -80°. Once all samples had been taken and processed, inflammatory protein measurements were performed using a proximity extension assay method (Olink Bioscience, Uppsala, Sweden) (389) with the Olink inflammatory panel.(390)

Olink

Proteins were assessed using the Olink® Target 96 inflammatory panel (Olink Proteomics AB, Uppsala, Sweden) in accordance with the manufacturer's guidelines. The technique employed, Proximity Extension Assay (PEA), has been extensively

detailed.(383) The technique employed, Proximity Extension Assay (PEA), has been extensively detailed. This method allows for the simultaneous analysis of 92 biomarkers using merely 1 µL of each sample. Through this approach, pairs of oligonucleotide-labelled antibody probes selectively bind to their respective protein targets. Upon proximity, these probes undergo hybridization, followed by a DNA polymerization event catalysed by DNA polymerase. Consequently, a distinct PCR target sequence is generated, which is then detected and quantified utilizing a microfluidic real-time PCR instrument (Biomark HD, Fluidigm). Quality control and normalization of Ct-data are ensured through the use of internal and external controls. The resulting assay read-out is presented as Normalized Protein eXpression (NPX) values, an arbitrary unit on a log2-scale where higher values correspond to elevated protein expression levels. The internal controls, which mimic and monitor various PEA steps, include two non-human proteins with matching antibody-probes serving as incubation or immune-controls, an IgG antibody with paired probes functioning as an extension control, and a complete double-stranded amplicon serving as a detection control. These internal controls are introduced to all samples alongside external controls, which include triplicates of negative controls for determining the limit of detection, as well as triplicates of inter-plate controls containing 92 sets of antibodies, each with matched probes for normalization purposes. NPX for each sample and assay is calculated using specific equations.(391)

1. $Ct_{(analyte)} - Ct_{(extension\ control)} = \Delta Ct_{(analyte)}$ (to decrease technical variation)
2. $\Delta Ct_{(analyte)} - Ct_{(median\ IPC)} = \Delta\Delta Ct_{(analyte)}$ (to improve inter plate variation)
3. $Correction\ factor_{(analyte)} - \Delta\Delta Ct_{(analyte)} = NPX_{(analyte)}$ (for more intuitive data).

The correction factor represents a predetermined variable unique to each assay and batch of reagents. Data quality assurance entails two steps: Firstly, the standard deviation is computed for each of the incubation or immune-controls and the detection control in every run. A run passes quality control only if the standard deviation for each control remains below 0.2. Secondly, each sample undergoes quality control using incubation control 2 and the detection control. The median of these controls for the run is calculated, and all samples within the run are compared

against it. Samples deviating more than +/- 0.3 NPX from the plate median concerning these two controls will fail quality control and receive a quality control warning in the output data file. Comprehensive assay validation data, including detection limits and intra- and inter-assay precision, are accessible on the manufacturer's website (www.olink.com).

Statistical analyses:

We computed the Spearman's correlation between 6-month mortality and 92 proteins from the Olink inflammatory panel. Results were adjusted for multiple testing using a False Discovery Rate correction.(392)

We compared results to an age matched cohort of 62 healthy individuals without injury (negative controls) and a cohort of 32 younger patients with traumatic fracture from the OPERA cohort (positive controls) and 19 prefrail and frail individuals without trauma using unpaired t-tests. Effect size of associations with mortality for clinical parameters and significant biomarkers was further evaluated using logistic regression analyses. All cohorts had the same panels run.

ROC curve analyses: Prediction models for 6-month mortality were performed on a nomogram combining age, sex and clinical indices fitted separately on frail and non-frail/pre-frail individuals from the whole hospital EHR cohort. The two logistic regression models fitted were:

Non frail and pre-frail hip fracture patients (CFS <4):

logit (180days_mortality) = -6.2463 -0.0161 ad_eGFR +0.2959 CCI +0.0991 NHFS +0.8045 CFS -0.2640 sex +0.0083 age

Frail hip fracture patients (CFS >=4):

logit (180days_mortality) = -6.1499 -0.0058 ad_eGFR + 0.170195 CCI +0.5061 CFS -0.6557 sex +0.0292 age

In addition, a hypothesis generating renal failure model including markers of renal function was generated but is outside the scope of this thesis For the FEMUR sub-cohort the ROC curve fitted in the frail group was also tested. In addition, three models including only circulating levels of FGF-23, IL15-RA plus eGFR-CYSC were also fitted. Finally, models including the clinical indices models plus each of the three biomarkers were also tested.

Analyses were performed using Python 3.11.1 (libraries pyplot, numpy and scipy), GraphPad Prism 10 and R 4.3.2 (library mediation).

7.6 Results

The descriptive characteristics of 59 frail fracture patients from the pilot study (FEMUR) who had serum samples taken immediately pre-operatively were compared to frail and non-frail hip fracture patients in the same hospital (Table 7.2). The FEMUR sub-cohort of frail patients with preoperative serum is broadly comparable to the overall patient population of frail hip fracture. After adjustment for a higher proportion of females and 3 years older age, there were no statistically significant differences between both groups in any clinical parameters. As expected, we observed highly significant differences in all clinical parameters between frail and non-frail patients.

	Hospital Records				Recruited frail. Sub-cohort		p-value frail vs non-frail EHRs	p-value frail EHR vs "FEMUR" sub-cohort
Frail category	Not frail or prefrail		Frail		FEMUR			
n	632		2230		59			
	Mean	SD	Mean	SD	Mean	SD		
Age (years)	78.91	8.41	85.23	8.02	88.34	5.93	<0.001	=0.003
Sex %F	66.93		70.27		84.75		0.0875	0.019
Clinical frailty score (CFS)	3.00	1.32	5.40	1.24	5.71	1.15	<0.001*	0.601*
Charlson Comorbidity Index (CCI)	4.67	1.76	6.27	1.97	5.78	1.54	<0.001*	0.051*
NHFS-mortality	5.25	2.96	8.91	5.30	8.20	4.79	<0.001*	0.107*
CKD%	13.13		28.21		33.90		<0.001*	0.558*
Admission eGFR %	73.92	17.01	64.23	20.56	61.85	19.70	<0.001*	0.998*
Length of Stay (LOS) days	12.99	8.46	18.02	12.87	19.48	13.07	<0.001*	0.299*
180-day mortality %	6.33		26.68		27.12		<0.001*	0.876*
Change in housing status %	21.99		46.10		40.68		<0.001*	0.258*

Table 7.2 Comparison between electronic health records (EHRs) for hip fracture surgery at Nottingham University Hospitals NHS Trust in the same period and the enrolled FEMUR sub-cohort. *p-value from logistic regression adjusted for age and sex.

When we assessed the correlation between 92 inflammatory proteins and 6-month mortality, thirteen proteins were apparently associated on univariate analysis. Of these, fibroblast growth factor 23 (FGF-23) and Interleukin-15 receptor alpha (IL15RA) passed a multiple testing correction (Figure 7.2).We compared levels of these markers in frail fracture patients to n=62 healthy controls (aged 42 ±11.57 years) , n=30 non-frail multiple rib fracture patients (aged 60±14.2 years, 9% female) and n=12 frail/prefrail individuals with no fracture (age 70.05± 7.16). The association between clinical traits and biomarkers with 6-month mortality post hip fracture surgery in the FEMUR cohort is shown in Table 7.3

Levels of FGF-23 were increased in non-frail trauma compared to healthy controls and in pre-frail individuals without fractures compared to healthy controls (Figure 7.3). In contrast, the pattern seen with IL-15RA (Figure 7.4) shows a significant decrease in pre-frail vs healthy controls and no difference in non-frail trauma compared to controls. Both markers have been implicated in kidney disease.(393, 394).

The value of these biomarkers in predicting mortality using ROC curve analyses was performed. It was found that the AUC for six-month mortality achieved with a model containing only clinical variables is similar for non-frail and frail patients overall and is not statistically significant in the FEMUR sub-cohort (Figure 7.5A) However, the positive predictive value (PPV) for this model is significantly higher in frail patients and in the FEMUR cohort than for non-frail patients (Figure 7.5B). In the cohort with preoperative biomarkers, each individual biomarker has a similar AUC and similar PPV to those of the clinical indices alone (Figures 7.5A and 7.5B). Although both the AUC and PPVs are higher combining clinical indices with biomarkers than biomarkers alone or clinical indices alone, there is no statistical significance between these values.

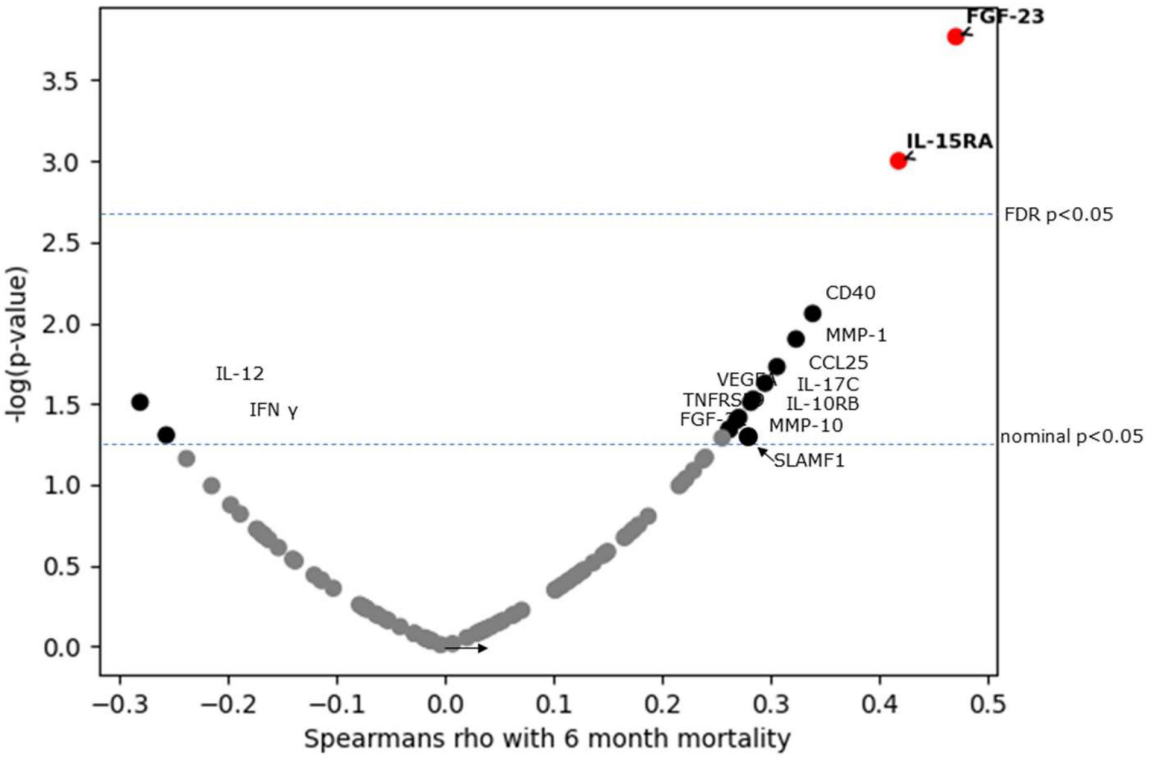


Figure 7.2 A volcano plot showing Spearman's correlation coefficient and -log p values for the association of 6-month mortality and molecular inflammatory markers. Two markers demonstrated a statistically significant false discovery rate p value of <0.05, FGF-23 and IL-15RA.

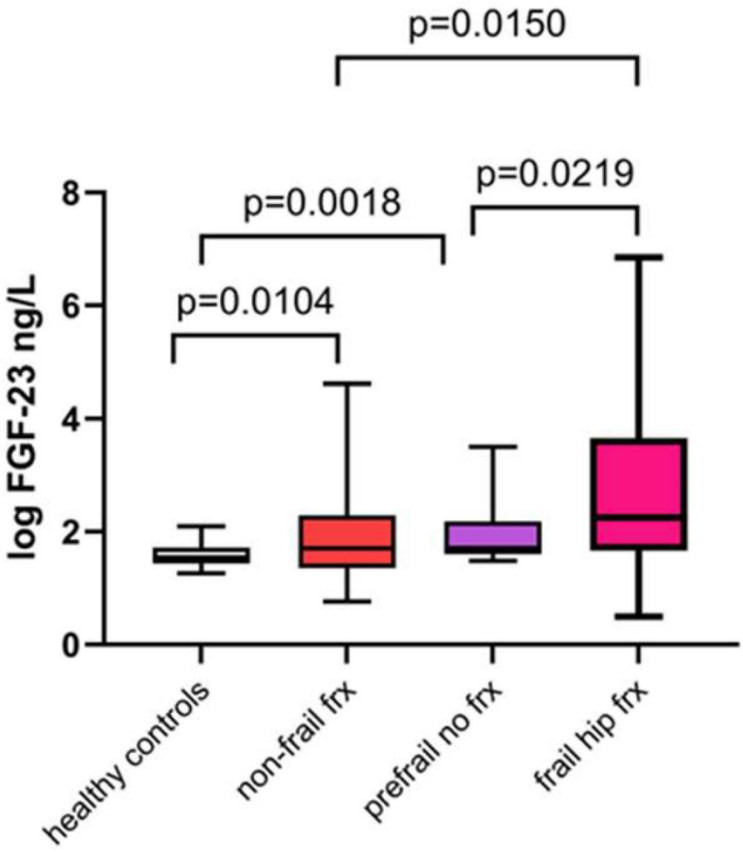


Figure 7.3. Boxplots showing the comparison of serum levels of FGF-23 in frail hip fractures with regards to healthy controls; non frail (impact) fracture cases and pre-frail individuals with no fractures. Boxplots: mean values of molecular marker levels indicated by plus signs and medians indicated by the bars in the boxes. The boxes show the interquartile range (IQR) and the whiskers extend from the boxes to indicate the 95% confidence interval.

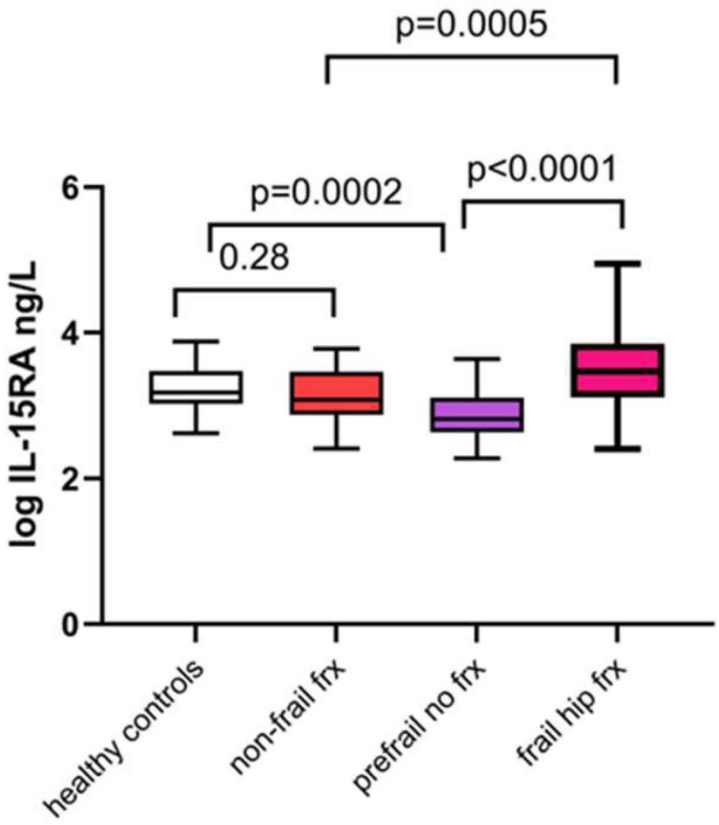


Figure 7.4. Boxplots showing the comparison of serum levels of IL15-RA in frail hip fractures with regards to healthy controls; non frail (impact) fracture cases and pre-frail individuals with no fractures. Boxplots: mean values of molecular marker levels indicated by plus signs and medians indicated by the bars in the boxes. The boxes show the interquartile range (IQR) and the whiskers extend from the boxes to indicate the 95% confidence interval.

	Died within 180d		Survived 180+d				
Clinical feature/biomarker	Mean	SD	Mean	SD	OR	95%CI	p-value
Age	89.71	4.79	86.90	6.16	1.11	0.98 to 1.27	0.09
Sex (F %)	88.2		83.3		1.50	0.28 to 8.08	0.64
Clinical frailty score (CFS)	6.47	0.87	5.40	1.11	3.08	1.44 to 6.61	3.3E-04
Nottingham hip fracture score (NHFS)	5.76	1.09	5.00	1.02	2.15	1.16 to 3.99	0.0127
Charlson comorbidity index (CCI)	7.06	1.14	5.40	1.18	2.73	1.52 to 4.94	3.3E-05
Renal disease at admission %	52.94		23.81		3.60	1.09 to 11.81	0.030
Admission eGFR	50.76	22.59	69.02	22.60	0.95	0.92 to 0.98	0.004
log FGF-23 ng/L	3.79	1.68	2.37	1.74	1.80	1.21 to t2.67	1.7E-04
log IL-15RA ng/L	3.85	0.48	3.39	0.50	4.90	1.52 to 15.81	0.001
Length of stay (LOS) days	21.53	18.62	18.93	12.20	1.01	0.97 to 1.06	0.800

Table 7.3. Association between clinical traits and biomarkers with 6-month mortality post hip fracture surgery in the FEMUR cohort.

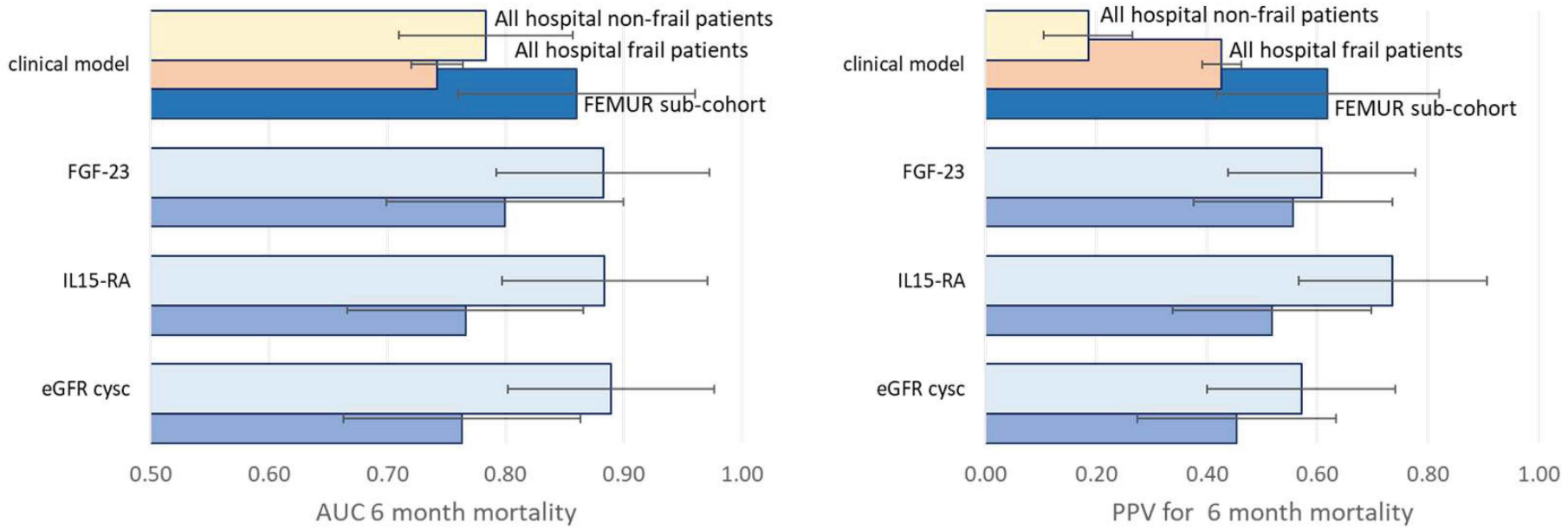


Figure 7.5 ROC curve analyses using clinical indices and biomarkers. **A** The area under the curve (AUC); and **B** positive predictive value (PPV) of biomarkers on 6-month postoperative mortality with 95% confidence intervals are shown. The clinical model includes a linear combination of age, sex, Charlsons' comorbidities index, clinical frailty score and Nottingham hip fracture score. The coefficients were fitted on all frail and non-frail patients from the hospital records (see Methods).

7.7 Discussion

In this exploratory study having tested 92 circulating inflammatory proteins, preoperative circulating levels of FGF-23 and IL-15RA were found to be significantly predictive of 6-month mortality post-surgery and of incident postoperative AKI after adjusted analysis. Having tested a large array of inflammatory markers, including various pro and anti-inflammatory cytokines; and growth factors, it is notable that only proteins with links to renal function were predictive of postoperative mortality.

Fibroblast growth factor (FGF)-23 is secreted by bone cells and is a key regulator of serum phosphate and active vitamin D3 levels.(393) In late-stage CKD, FGF-23 cannot reduce serum phosphate levels, and high FGF-23 concentrations result in left ventricular hypertrophy (LVH), faster CKD progression, and mortality.(395) FGF-23 is an independent predictor for LVH adjusting for eGFR,(396, 397) and appears to be the molecular link between the bone's physiological response to trauma (398, 399) and acute kidney injury (399) or an exacerbation of renal insufficiency in frail individuals.(398) FGF-23 has also been identified as a predictor of AKI and death following cardiac surgery.(400)

IL-15RA mediates pleiotropic proinflammatory signals involved in several inflammatory and cardiovascular disorders.(401) IL-15RA was identified (394) as one of 17 proteins involved in 10-year risk of end-stage renal disease in two large US cohorts of individuals with type 2 diabetes. It is also one of 21 proteomic biomarkers associated both with kidney function decline and incident CKD over a 13-year period in a large longitudinal German study.(402)

Of interest the data presented here also show that circulating levels of IL-15RA are predictive of AKI. We also report the novel finding that this molecule can be used as a predictor of postoperative mortality in hip fracture patients, presumably via its effects on cardiorenal disease.

7.7.1 Reflections and Limitations

The current study has several strengths. The pilot sub-cohort analysed for biomarkers is comparable in clinical and demographic characteristics to a much larger cohort of frail hip fracture patients from the largest trauma centre in the UK, suggesting that the data from our study should be generalizable to the wider hip fracture populations. We were able to compare the biomarkers tested to a series of relevant positive and negative controls, namely healthy controls, non-frail severe fractures and non-fractured prefrail and frail individuals. Through the analysis of EHRs we were also able to compare the predictive value of biomarkers for mortality to that of combined clinical and demographic indices fitted in the large database.

The key limitation of the present study is the pilot nature of the data, i.e. the sample size of fracture cases with preoperative serum. This has been compensated for by ensuring that key clinical parameters are comparable to those in the larger frail hip fracture patient population from the largest Major Trauma Centre in England, and comparison to appropriate positive and negative controls.

7.7.2 Conclusion

In conclusion, this is an exploratory study investigating inflammatory mediators of high postoperative mortality in hip fracture patients identified renal-related factors as strong predictors of mortality. Our data suggest that either FGF-23 or IL-15RA or both should be assessed as potential biomarkers to make accurate clinical decisions regarding risk of postoperative AKI, and to evaluate the risk of mortality and complications in frail hip fracture patients.

8 Overall conclusion

With increasing frailty patients had a poorer global health state at all time points following hip fracture. In lower frailty, patients' health state did improve but not as rapidly and did not return to pre-injury levels. In patients with severe frailty however there was no improvement. This observation held true for subjective and objective measures of muscle performance. Specifically, the timed up and go test was a good binary predictor for successful discharge home for patients admitted from their own home. Outcomes mainly differed at the extreme spectrum of frailty rather than a graduated change over frailty status.

This study has found when stratified for frailty there is a significant differential gene expression in the Vastus Lataralis muscle of the fractured leg which is not evident in the non-fractured leg. This suggests a differential localised physiological response to trauma. As clinical frailty increases the inhibition of apoptosis and cell death decreases, and the activation of metabolism of protein and catabolism increases. The most significant changes were seen between the extreme ends of frailty. This study has also shown this to be true where the most significant differences in subjective and objective measures of muscle function and performance were also between the extreme ends of frailty spectrum. Therefore, it could be inferred that frailty from a deep phenotype model does mirror to an extent a deficit model with worsening outcomes with increasing frailty. Factors to promote anti-apoptosis and cell death with prevention of protein catabolism within muscle may be therapeutic targets for interventions to improve frailty pre and post hip fracture.

Two systemic inflammatory markers were found to be predictive of 6-month mortality post hip fracture surgery and in the incidence of post operative acute kidney injury after adjusted analysis. Only proteins with links to renal function were predictive of post operative mortality. FGF-23 appears to be the molecular link between the bone's physiological response to trauma and acute kidney injury or exacerbation of renal insufficiency. This highlights the importance of the assessment and management of

renal function before during and after hip fracture surgery, and the prospect of targeting this biomarker to improve outcomes for patients.

8.1 Further Work

8.1.1 Subjective and objective measures of muscle performance

The EQ5D index appears to be a useful objective measure of global health assessment, following the results from this study, further work would need to investigate if there is a specific component of the EQ5D which is most affected following a hip fracture which impacts differently amongst differing frailty groups. Although in the NHS the use of the CFS has become mainstream it is difficult to appreciate changes in frailty states for patients with hip fracture since they are not routinely followed up. Therefore, there is an argument to be made for the use of subjective frailty assessment tools for example the GFI to help evaluate changes in frailty to show impact of changing care pathways or treatment but firstly this would need to be validated in this cohort of patients. This study has shown variation in mobility recovery following a hip fracture based on different admission frailty states. Further work is needed to ascertain if comorbidities and post operative complications affect the mobility in the severely frail and if the surgical approach during hip fracture surgery plays a role in mobility post-op with regards to muscle splitting and sparing techniques especially in those with low functional reserve such as the severely frail. To improve mobility outcomes for those with mild to moderate frailty further investigation into the first week of recovery is needed with a view to optimise rehabilitation strategies such as an enhanced recovery or intensive initial phase of therapy in the first week following surgery or prolonged/intensive community physio to improve mid to long term mobility outcomes.(403) This could also apply to the ability to perform the TUG test and TUG time, and further evaluation of the TUG at day 90 would also be a useful measure. Other factors such as nutritional assessment and dietary intake although not assessed as part of this study may also need to be investigated as part of this.

Although functional assessment is key for patients this must be performed by a trained individual and can be labour intensive with time and resource allocation. Grip strength

as a snapshot measure could be used as a surrogate but this study has shown further work may be needed to ascertain if the timing of grip strength assessment impacts the values obtained and if existing hip fracture rehabilitation influences grip strength scores. US thickness of vastus lateralis would need further evaluation with respect to the D3 creatine dilution assessment since this was obtained in some of the consented patients. However, due to time constraints secondary to the covid-19 pandemic this is not included as part of this thesis.

A combined approach utilising comprehensive occupational therapy review through the BADL assessment, and physiotherapy assessment utilising grip strength and TUG test may provide a unique opportunity to enhance risk stratification and enhance recovery programs tailored to specific frailty groups, to prevent mortality in high frailty groups and improve functional independence in lower frailty groups. Since the national standardisation of hip fracture care is currently one size fits all perhaps findings from this study may lead onto a review of the goals of hip fracture treatment specifically in the frail population. No longer is mortality and prevention of post operative complications the main agenda but retention of muscle functional performance and independence now become more evident and increasingly important for an aging population.

8.1.2 Muscle gene expression

We have shown that undertaking basic science research on patients who are as frail as those who have sustained a hip fracture is possible with correct planning and forethought to ensure patient care is upheld and additional distress in those who are cognitively impaired is minimised. Another major advantage of this study to be able to recruit patients who do not have capacity through an appropriate consultee, and the ability to obtain tissue samples from participants in theatre in a controlled sterile environment under anaesthesia already being administered for their hip fracture treatment.

Based upon the differential gene expression analysis and identification of cellular functions occurring within the damaged limb across frailty groups. The next step is to analyse in which cells the genes are being expressed through immunohistochemistry and immunofluorescence. Following this further work can be completed to investigate if the expressed genes are leading to the formation of proteins through translation. Although this was in part an objective of the FEMUR study it was not completed due to time constraints and lack of research staff due to the Covid-19 pandemic. However, this is still achievable within the same cohort of participants who have already been recruited, due to the quality and quantity of the tissue samples obtained and currently being stored.

Further research is required to establish a mechanistic link between apoptosis and muscle atrophy, identify the most relevant apoptotic pathway(s) to target, and determine the optimal timing for intervention. Studies are also needed to investigate whether the network arrangement of mitochondria facilitates the propagation of the apoptotic signalling under severe muscle-atrophying conditions. The occurrence of such an amplification could provide a mechanistic explanation for the higher degree of atrophy experienced by older adults during periods of protracted disuse (e.g., prolonged bed rest).

8.1.3 Inflammatory marker proteomic analysis

Importantly, the strongest marker of incident AKI in our study was FGF-23, followed by IL-15RA. We show here that a substantial proportion of the effect of these biomarkers is mediated by the postoperative incidence of AKI. If these biomarkers were validated in larger study samples, this would be a strong incentive to incorporate such measures within clinical practice to improve patient care. We also find that, along with FGF-23 and IL-15RA, eGFR of cystatin C is also much more informative of AKI incidence than creatinine derived eGFR. Future studies should focus on defining the health economic benefits of obtaining more reliable predictors of post-operative AKI incidence in this patient population and on exploring how to improve clinical

management to reduce the incidence of AKI and therefore also post-operative mortality.

8.2 Concluding remark

This first ever study investigating the biological basis of frailty in patients with hip fracture has shown major key findings of the effect of frailty on post operative muscle function, performance, and survival. It has identified multiple potential avenues for further research into potential therapeutic interventions to improve outcomes.

9 Appendices

9.1 Appendices A Skeletal muscle assessment, function and contributions to outcomes

9.1.1 Questionnaire example





FEMUR Study

Day 7 Questionnaires

Participant ID : _____

Completion Date : __/___/____

Clinical Frailty Score : _____

Completion:
Information can be obtained from the patient's self-report, from a separate party who is familiar with the patient's abilities (such as a relative, or ward staff), or from observation.

For each question, please indicate the answer that most closely corresponds to the patient's level of ability or response. This should reflect ability prior to the fall rather than current functioning.

As you move through the questionnaire sets, you will notice some repetition in the questions being asked. To reduce burden to the participant or separate party, where questions are identical, these can be completed from previous responses

Completed By: _____

Date: __/___/____

Office use only:

Summary of Performance Status:

- ☐ Normal activity without restriction.
- ☐ Restricted in physically strenuous activity but ambulatory and able to carry out light work.
- ☐ Ambulatory and capable for all self-care, unable to carry out any work and about >50% of waking hours.
- ☐ Capable only of limited self-care, confined to bed or chair and about <50% of waking hours.
- ☐ Completely disabled, cannot carry on any self-care, totally confined to bed or chair.

Version 1.0 14.05.2020

The Rivermead Mobility Index

	YES	NO
Turning over in bed: Do you turn over from your back to your side without help?		
Lying to sitting: From lying in bed, do you get up to sit on the edge of the bed on your own?		
Sitting balance: Do you sit on the edge of the bed without holding on for 10 seconds?		
Sitting to standing: Do you stand up from any chair in less than 15 seconds and stand there for 15 seconds, using hands and/or an aid if necessary?		
Standing unsupported: (If possible, ask to stand) Observe standing for 10 seconds without any aid		
Transfer: Do you manage to move from bed to chair and back without any help?		
Walking inside: (with an aid if necessary): Do you walk 10 meters, with an aid if necessary, but with no standby help?		
Stairs: Do you manage a flight of stairs without help?		
Up and down four steps: Do you manage to go up and down four steps with no rail, but using an aid if necessary?		
Walking outside: (even ground): Do you walk around outside, on pavements, without help?		
Walking outside: (uneven ground): Do you walk over uneven ground (grass, gravel, snow, ice etc) without help?		
Walking inside: (with no aid): Do you walk 10 meters inside, with no caliper, splint, or other aid (including furniture or walls) without help?		
Picking up off floor: Do you manage to walk five meters, pick something up from the floor, and then walk back without help?		
Running: Do you run 10 meters without limping in four seconds (fast walk, not limping, is acceptable)?		
Bathing: Do you get into/out of a bath or shower and to wash yourself unsupervised and without help?		

Office use only: **Calculated Index Score =**

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Groningen Frailty Index

	YES	NO
Mobility. *Can the patient perform the following tasks without assistance from another person (walking aids such as a can or a wheelchair are allowed)*		
1. Grocery shopping		
2. Walk outside house (around house or to neighbour)		
3. Getting (un)dressed		
4. Visiting restroom		
Vision 5. Does the patient encounter problems in daily life because of impaired vision?		
Hearing 6. Does the patient encounter problems in daily life because of impaired hearing?		
Nutrition 7. Has the patient unintentionally lost a lot of weight in the past 6 months (6kg in 6 months or 3kg in 3 months)?		
Co-morbidity 8. Does the patient use 4 or more different types of medication?		

	YES	NO	Sometimes
Cognition 9. Does the patient have any complaints on his/her memory (or diagnosed with dementia)?			
Psychosocial 10. Does the patient ever experience emptiness around them? e.g. You feel so sad that you have no interest in your surroundings.			
11. Does the patient ever miss the presence of other people around him? Or do you miss anyone you love?			
12. Does the patient ever feel left alone? e.g. You wish there was someone to go with you for something important.			
13. Has the patient been feeling down or depressed lately?			
14. Has the patient felt nervous or anxious lately?			

Physical Fitness

15. How would the patient rate his/her own physical fitness (between 0-10)? _____

Office use only: **Calculated Index Score =**

Version 1.0 14.05.2020

Barthel Index of Activities of Daily Living

Toileting:

Bowels

- ☐ incontinent (or needs enemas)
- ☐ occasional accident (once/week)
- ☐ continent

Toilet Use

- ☐ dependent
- ☐ needs some help, but can do something alone
- ☐ independent (on and off, dressing, wiping)

Bladder

- ☐ incontinent, or catheterized and unable to manage
- ☐ occasional accident (max. once per 24 hours)
- ☐ continent (for over 7 days)

Personal Care:

Grooming

- ☐ needs help with personal care
- ☐ independent face/hair/teeth/shaving (implements provided)

Bathing

- ☐ dependent
- ☐ independent (or in shower)

Dressing

- ☐ dependent
- ☐ needs help, but can do about half unaided
- ☐ independent (including buttons, zips, laces, etc.)

Feeding

- ☐ unable to feed unaided
- ☐ needs help cutting, spreading butter, etc.
- ☐ independent (food provided within reach)

Mobility:

Mobility

- ☐ immobile
- ☐ wheelchair independent, including corners, etc.
- ☐ walks with help of one person (verbal or physical)
- ☐ independent (but may use any aid, e.g., stick)

Transfer

- ☐ unable – no sitting balance
- ☐ major help (one or two people, physical), but can sit
- ☐ minor help (verbal or physical)
- ☐ independent

Stairs

- ☐ unable
- ☐ needs help (verbal, physical, carrying aid)
- ☐ independent up and down

Office use only: **Calculated Index Score =**

Version 1.0 14.05.2020

EQ-5D Health Questionnaire

Under each heading, please tick the ONE box that best describes the participant's responses about how they feel about their health TODAY.

MOBILITY

- ☐ I have no problems in walking about
- ☐ I have slight problems in walking about
- ☐ I have moderate problems in walking about
- ☐ I have severe problems in walking about
- ☐ I am unable to walk about

USUAL ACTIVITIES *(e.g. work, study, housework, family or leisure activities)*

- ☐ I have no problems doing my usual activities
- ☐ I have slight problems doing my usual activities
- ☐ I have moderate problems doing my usual activities
- ☐ I have severe problems doing my usual activities
- ☐ I am unable to do my usual activities

SELF-CARE

- ☐ I have no problems washing or dressing myself
- ☐ I have slight problems washing or dressing myself
- ☐ I have moderate problems washing or dressing myself
- ☐ I have severe problems washing or dressing myself
- ☐ I am unable to wash or dress myself

PAIN / DISCOMFORT

- ☐ I have no pain or discomfort
- ☐ I have slight pain or discomfort
- ☐ I have moderate pain or discomfort
- ☐ I have severe pain or discomfort
- ☐ I have extreme pain or discomfort

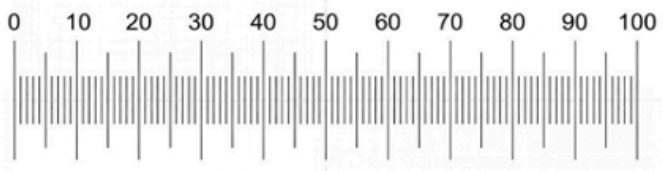
ANXIETY / DEPRESSION

- ☐ I am not anxious or depressed
- ☐ I am slightly anxious or depressed
- ☐ I am moderately anxious or depressed
- ☐ I am severely anxious or depressed
- ☐ I am extremely anxious or depressed

We would like to know how good or bad your health is TODAY.

This scale is numbered from 0 to 100. Please place a vertical line along this ruler to indicate how you rate your health; 100 means the best health you can imagine, 0 means the worst health you can imagine.

worst health you can imagine



best health you can imagine

Office use only: **Calculated Index Score =**

Version 1.0 14.05.2020

9.1.2 Results – Subjective and Objective measures of quality of life, muscle function and performance.

9.1.2.1 Global rating of function EQ5D

Descriptive statistics

We obtained complete EQ5D-index and EQ5D-health data as demonstrated in Table 9.1. The mean EQ5D-index was 0.238 and 0.499 at day 7 and day 9 respectively and the mean EQ5D-health was 51.59 and 60.39 at day 7 and 90 respectively. Histograms and skewness z test showed a normal distribution of data, Figures 9.1-9.4 and Table 9.1.

Observing the overall EQ5D data paired t-tests revealed the EQ5D-index at day 90 (M=0.499, SD=0.339) was statistically significantly greater than day 7 (M=0.234, SD=0.300), t(73) = 6.383, p<.001. However there was no statistical significant difference in EQ5D-health between day 7 (M=54.58, SD=23.012) and day 90 (M=60.60, SD=21.211), t(71) = 1.880, p=0.064.

The relationship between HGS and EQ-5D

There is a significant weak positive relationship between HGS and EQ5D-index and health on day 7, and day 90 (Table 5.5) (Figures 9.5-9.8).

The EWGSOP2 demonstrates cut-offs for low grip strength for men and women. Low grip strength is one of the hall marks for a diagnosis of sarcopenia and ameliorates the difference between male and female patients since it takes this into account in determining the cut-off values. Therefore, we evaluated the relationship of low grip strength with EQ5D-index and EQ5D-health.

There was a significant weak negative correlation between grip strength according to EWGSOP2 cut-offs and EQ5D-index on day 7, and day 90. There was a significant moderate negative correlation between grip strength according to EWGSOP2 cut-offs and EQ5D-health on day 7, and weak negative correlation at day 90 (Table 5.5).

Figures 9.9 and 9.10 show patients who have a normal grip strength have a greater mean EQ5D-index value then those who have a low grip strength at day 90. But, at day 7 it appears to be similar. Patients with normal grip strength appear to have a greater mean EQ5D-health at day 7 and day 90 than those with a low grip strength.

Patients with a normal grip strength have a statistically significant greater EQ5D scores than those with a low grip strength as shown in Table 9.2. Patients with a normal grip strength have a greater improvement in their EQ5D-index than those with a low grip strength from day 7 to day 90. However, there is not a statistically significant improvement in EQ5D-health (Table 9.3).

The relationship between TUG test and the EQ-5D

The ability to perform a TUG test shows a significant positive correlation with EQ5D. This was moderate for EQ5D-index on day 7 and weak for EQ5D-index at day 90 and EQ5D-health (Table 5.5). There was a significant weak negative correlation between TUG time and EQ5D-index and health (Table 5.5) (Figures 9.11 – 9.14).

Figures 9.15 and 9.16 shows patients who can complete the TUG test have a greater mean EQ5D-Index than those unable to perform the test both at day 7 and 90. Patients who are able to complete the TUG test at day 7 have a similar mean EQ5D-index at day 7 than those who are unable to complete the test at day 90. There does not appear to be any difference in mean EQ5D-health scores from day 7 to 90 irrespective of the ability to complete the TUG test.

Independent t-test confirmed patients who can complete a TUG test have a statistically significant greater mean EQ5D-Index and EQ5D-health scores compared with those who are unable to complete the test (Table 9.4). All patients have an improvement in EQ5D-index scores whether they can complete the TUG test or not with the mean rate of change similar for both groups. There is no statistically significant improvement in EQ5D-health based upon ability to complete the TUG test (Table 9.5).

The relationship between VLT and EQ-5D

There was no statistically significant correlation between EQ5D-Index and health with VLT-F, and no statistically significant correlation between EQ5D-index and VLT-NF. There was a statistically significant weak positive correlation between EQ5D-health and VLT-NF (Table 5.5).

9.1.2.2 Groningen frailty index

Descriptive statistics

We obtained complete GFI data as demonstrated in Table 9.1. The mean GFI was 7.40 7.97 and 7.16 at pre-injury, day 7, and day 9 respectively. Histograms and skewness z test showed a normal distribution of data (Figures 9.17-9.19 and table 9.1).

Observing the overall data, paired sample t-test demonstrated the day 7 GFI (M=7.97, SD=2.286) was statistically significantly higher than the pre-injury GFI (M=7.38, SD=2.707), t(132) = -2.272, p=0.025. There was no statistical significant difference between the day 90 GFI (M=7.16, SD=3.107) when compared with the pre-injury GFI (M=7.26, SD=2.863), t(75) = 0.375, p=0.709. The day 90 GFI (M=7.16, SD=3.107) was significantly lower than the day 7 GFI (M=8.09, SD=2.210), t(75) = 2.732, p=0.008.

The relationship between HGS and GFI

Pearsons correlation showed a significant weak negative relationship between HGS and pre-injury GFI, and day 90 GFI. There was no significant correlation between HGS and day 7 GFI (Table 5.5) (Figures 9.20 – 9.22).

Spearmans correlation demonstrated a weak positive correlation between low grip strength according to EWGSOP2 and pre-injury GFI, and day 90 GFI, There was no statistically significant correlation between low grip strength and day 7 GFI (5.5 and Figure 9.23). This was confirmed by independent samples t-test demonstrating patients with a low grip strength had a statistically significant greater GFI pre-injury and at day 90, but no statistically significant difference at day 7 (Table 9.2).

In patients with normal grip strength paired t-test showed no statistically significant difference between the day 7 and the pre-injury GFI, and the day 90 and pre-injury GFI. The day 90 GFI was statistically significantly lower than the day 7 GFI (Table 9.6). In patients with low grip strength there was no statistically significant difference between any of the GFI scores at any stage.

The relationship between TUG and GFI

Both Spearmans and Pearsons correlation showed no statistically significant correlation between the ability to complete the TUG and GFI, and TUG time and GFI respectively, at any time point. (Table 5.5).

The relationship between VLT and GFI

Pearsons correlation demonstrated a statistically significant weak negative correlation between VLT-F and GFI pre-injury but no statistically significant correlation with GFI on day 7, or day 90. There was a statistically significant weak negative correlation between VLT-NF and GFI pre-injury and day 90, but no statistically significant correlation with day 7 GFI (Table 5.5).

9.1.2.3 Subjective measure of function Rivermead mobility index

Descriptive statistics

Complete RMI data is demonstrated in Table 9.1. The mean RMI was 10.11, 3.06 and 7.82 at pre-injury, day 7 and day 90 respectively. Histograms and skewness z test showed a normal distribution of data at day 90 only. Pre-injury and day 7 RMI showed a non-normal distribution, as shown in Figures 9.24– 9.26.

Paired t-tests showed, the day 7 RMI (M=3.06, SD=2.433) was statistically significantly lower than the pre-injury RMI (M=10.14, SD=3.482), t(132) = 25.790, p<.001. The day 90 RMI (M=7.82, SD=4.217) was statistically significantly lower than the pre-injury RMI (M=10.83, SD=3.139), t(75) = 7.735, p<.001. The day 90 RMI (M=7.82, SD=4.217) was statistically significantly higher than the day 7 RMI (M=3.30, SD=2.598), t(75) = -10.879, p<.001. Overall following hip fracture, the RMI decreased following injury but then increased during recovery but did not return to pre-injury levels.

The relationship between HGS and RMI

Spearmans correlation showed a negative correlation between low grip strength (according to EWGSOP2) and RMI. This was moderate at pre-injury and weak at day 7 and 90 Pearsons correlation demonstrated a significant positive relationship between HGS and RMI. This was moderate at pre-injury and day 7, and weak at day 90 (Table 5.5 and Figures 9.27 – 9.29)

Figure 9.30 shows patients with a low grip strength according to EWGSOP2 cut-offs have a lower mean pre-injury, day 7 and day 90 RMI (Table 9.2) compared with those with a normal grip strength. This was confirmed with independent samples t-test.

At day 7 these differences may be less significant. RMI does not appear to return to pre-injury levels based upon grip strength. Observing patients with normal and low grip strength paired sample t-test showed a significant statistical difference between RMI scores at pre-injury, day 7 and day 90. Irrespective of grip strength all patients had a drop from pre-injury to day 7, and all subsequently improved but not to pre-injury levels (Table 9.6).

The relationship between TUG test and RMI

Patients who attempted the TUG but were unable to complete the test had a time of 999seconds recorded. Spearmans correlation showed positive correlation between ability to perform the TUG test and RMI. The correlation is weak at pre-injury and moderate at day 7 and 90. Pearsons correlation shows a negative correlation between TUG time and RMI. This was weak with pre-injury and moderate with day 7 and 90 (Table 5.5 and Figures 9.31 – 9.33).

Figure 9.34 shows patients able to complete the TUG test have a greater pre-injury, day 7 and day 90 RMI than those who are unable to complete the test. In both groups of patients. This is confirmed by an independent samples t-test showing a statistically significant difference between the ability to complete a TUG test and the pre-injury, day 7 and day 90 RMI scores (Table 9.7) Visual inspection shows the RMI does not return to pre-injury levels, which is confirmed by paired sample t-test showing a

significant statistical difference between RMI scores and ability to complete the TUG test. Irrespective of being able to complete the TUG test, all patients had a drop from pre-injury to day 7, and all subsequently improved but not to pre-injury levels (Table 9.8).

The relationship between VLT and RMI

Pearsons correlation demonstrated no statistically significant difference between VLT-F and RMI at any time point. There was a statistically significant weak positive correlation between VLT-NF and RMI at all time points (Table 5.5).

9.1.2.4 Objective measure of function Barthel activity of daily living

Descriptive statistics

Complete BADL data is demonstrated in Table 9.1. The mean BADL was 85.11, 47.89 and 70.58 at pre-injury, day 7 and day 90 respectively. Histograms and skewness z test showed a normal distribution of data at day 7 only. Pre-injury and day 90 BADL showed a non-normal distribution, as shown in Figures 9.35 – 9.37.

Paired sample t tests showed day 7 BADL (M=47.89, SD=23.981) was statistically significantly lower than the pre-injury BADL (M=85.08, SD=19.829), t(132) = 22.308, p<.001. The day 90 BADL (M=70.58, SD=29.390) was statistically significantly lower than the pre-injury BADL (M=89.03, SD=16.223), t(76) = 7.179, p<.001. The day 90 BADL (M=70.58, SD=29.390) was statistically significantly higher than the day 7 BADL (M=52.92, SD=22.2568), t(7) = -6.446, p<.001.

The relationship between HGS and BADL

Pearsons correlation demonstrated a significant positive correlation between HGS and BADL. This was a strong for pre-injury and day 7, and moderate for day 90 (Table 5.5 and Figures 9.38 – 9.40). Spearmans correlation shows a significant moderate negative correlation between low grip strength (according to EWGSOP2 definition) and BADL at all time points (Table 5.5).

Inspecting the data visually in Figure 9.41 patients with a low grip strength according to EWGSOP2 cut-offs have a lower BADL at all time points when compared with those with a normal grip strength. In patients with a low grip strength there is overlap of the confidence intervals between day 7 and day 90 BADL suggesting limited statistically significant difference. However, for patients with a normal grip strength there is no overlap. No patient returns to their pre-injury BADL score irrespective of grip strength.

Independent samples t-test confirmed the visual inspection of Figure 9.41 it showed patients with a low grip strength had a statistically significant lower BADL at pre-injury, day 7 and day 90 than those with normal grip strength (Table 9.2).

Paired sample t-test showed in both low and normal grip strength groups there is a statistically significant drop in BADL from pre-injury to day 7 which then improves at day 90, but not to pre-injury levels (Table 9.6).

The relationship between TUG and BADL

Spearmans correlation showed a significant positive correlation between the ability to perform the TUG test and BADL. This was a moderate at pre-injury and day 90 but strong at day 7. Pearsons correlation demonstrated a significant negative correlation between TUG time and BADL, this was moderate for pre-injury and strong for day 7 and 90 (Table 5.5 and Figures 9.42 – 9.44).

Figure 9.45 shows patients unable to perform a TUG test had a lower pre-injury, day 7 and day 90 BADL than those able to perform a TUG test. This was confirmed to be statistically significant by independent samples t-test (Table 9.7).

In both groups able and unable to complete TUG test, paired sample t-test showed there is a statistically significant drop in BADL from pre-injury to day 7 which then improves at day 90 but does not return to pre-injury levels. There is a greater improvement in BADL score in patients who can complete the TUG test (Table 9.8)

The relationship between VLT and BADL

Pearson's correlation showed a statistically significant weak positive correlation between VLT-F and BADL pre-injury and day 7. There was no statistically significant correlation with BADL at day 90. There was a statistically significant weak positive correlation between VLT-NF and BADL at all time points (Table 5.5 and Figures 9.46-9.50).

9.1.2.5 Muscle mass assessment Ultrasound measurements of vastus lateralis thickness

Descriptive statistics

Complete ultrasound data is demonstrated in Table 9.1. The mean VLT-F thickness was 2.695cm and the mean VLT-NF was 2.444cm. Histograms and skewness z test showed a normal distribution of data (Figures 9.51 and 9.52). Missing measurements were due to surgical dressings obscuring the field of view in the fractured limb and patients becoming deceased prior to ultrasound measurements being performed.

Pearsons correlation showed a significant moderate positive correlation between VLT-F and VLT-NF (Table 5.10).

The relationship between HGS and VLT

Pearsons correlation showed a weak positive correlation between both VLT-F and VLT-NF with hand grip strength. Spearmans correlation showed a weak negative correlation between low grip strength (EWGSOP2) and both VLT-F and VLT-NF (Table 5.10).

Figure 9.53 shows patient with a low grip strength have a thinner vastus lateralis muscle thickness in both the fractured leg and non-fractured leg when compared with those with a normal grip strength. It also shows a larger difference between legs when compared with patients with a normal grip strength. This is confirmed by independent samples t-test. (Table 9.2)

The relationship between TUG and VLT

Spearmans correlation showed no correlation between VLT-F and VLT-NF and the ability to undertake a TUG test. Pearsons correlation demonstrated no correlation between VLT-F and VLT-NF with TUG time (Table 5.10).

9.1.2.6 Muscle performance assessment Timed up and go test.

Descriptive statistics

The number of patients were eligible to undertake the TUG test was 117. Missing measurements were due to patients becoming deceased, and unsafe to perform the test either due to medical complications or balance problems. The mean overall TUG time was 604.57s (Table 9.1) but this is where we have assigned those who could not finish the TUG test a time of 999s. When observing the patients who managed to complete the tug test n=51, the mean TUG time was 94.12s (SD 60.077). Only one patient completed the TUG test in under 20s which is the cut off for the EWGSOP2 sarcopenia measurement.

Spearmans correlation demonstrated a very strong negative correlation between TUG time and ability to complete TUG test r(115) = -.948, p<.001 (Table 5.10). Histograms and skewness z test showed a normal distribution of data as shown in Figures 9.54 and 9.55.

The relationship between HGS and TUG time

Spearmans correlation demonstrated a statistically significant weak positive correlation between HGS and ability to perform the TUG test and Pearsons correlation showed a weak negative correlation with TUG time (Table 5.10. and Figure. 9.56)

Figure 9.57 shows those with a low grip strength have a greater TUG time compared with those with a normal grip strength. This was confirmed with independent samples t-test (Table 9.2) Chi squared test (Table 9.9) demonstrated an association between ability to perform TUG test and low grip strength. $X^2(1)=20.136$, <.001 (Table 9.10)

9.1.2.7 Muscle strength assessment Hand grip strength

Descriptive statistics

Complete grip strength data is demonstrated in Table 9.1. The mean grip strength was 17kg. Histograms and skewness z test showed a normal distribution of data (Figure 9.58 Grip strength data was collected for 133 patients, missing data was due to deceased patients, or declining or missed undertaking the assessment. 68 patients had a low grip strength according to EWGSOP2 definition. 110 were right-handed and 22 had concomitant known hand pathology. 126 patients undertook the test in a seated position while 7 patients undertook the assessment in bed but in the seated cardiac chair position. Spearmans correlation showed a strong negative correlation between HGS and low grip strength as measured by EWGSOP2 Table 5.10.

9.1.2.8 Table of results

Table 9.1 Descriptive statistics for Euro-Qol health-related quality of life (EQ-5D), subjective measures of frailty Groningen frailty index (GFI), and subjective and objective measures of muscle function Rivermead mobility index (RMI), Barthels activity of daily living (BADL), hand grip strength, timed up and go test, mid-thigh Vastus Lateralis muscle thickness). Abbreviations n=number; SD=standard deviation; SE=standard error. VLT-F=Vastus lateralis thickness fractured limb; VLT-NF= Vastus lateralis thickness non-fractured limb; TUG=timed up and go test; HGS= Hand grip strength.

	n	Range	Min	Max	Mean		SD	Skewness			Kurtosis		
					Value	SE	Value	Value	SE	Z	Value	SE	Z
EQ5D-Index													
Day 7	132	1.594	-.594	1.000	0.238	0.026	0.304	0.020	0.211	0.095	-0.348	0.419	-0.831
Day 90	74	1.510	-.510	1.000	0.499	0.039	0.339	-0.769	0.279	-2.756	-0.096	0.552	-0.174
EQ5D-Health													
Day 7	128	100	0	100	51.6	2.0	22.8	-0.215	0.214	-1.005	-0.281	0.425	-0.661
Day 90	74	90	10	100	60.4	2.5	21.2	-0.305	0.279	-1.093	-0.487	0.552	-0.882
GFI													
Pre-Injury	139	13	1	14	7.4	0.2	2.8	0.081	0.206	0.393	-0.381	0.408	-0.934
Day 7	133	10	3	13	8.0	0.2	2.3	0.250	0.210	1.190	-0.520	0.417	-1.247
Day 90	76	13	1	14	7.2	0.4	3.1	0.131	0.276	0.475	-0.808	0.545	-1.483

	n	Range	Min	Max	Mean		SD	Skewness			Kurtosis		
					Value	SE	Value	Value	SE	Z	Value	SE	Z
RMI													
Pre-Injury	139	14	1	15	10.1	0.3	3.5	-0.721	0.206	-3.500	-0.156	0.408	-0.382
Day 7	133	10	0	10	3.1	0.2	2.4	0.781	0.210	-3.719	0.085	0.417	0.204
Day 90	76	15	0	15	7.8	0.5	4.2	-0.063	0.276	-0.228	-0.935	0.545	-1.716
BADL													
Pre-Injury	139	85	15	100	85.1	1.7	19.6	-1.580	.206	-7.700	1.777	0.408	4.355
Day 7	133	100	0	100	47.9	2.1	24.0	-0.139	.210	-0.662	-0.724	0.417	-0.174
Day 90	77	100	0	100	70.6	3.3	29.4	-0.927	.274	-3.380	-0.438	0.541	-0.810
Mean VLT-F (cm)	134	2.695	0.802	3.497	1.755	0.045	0.520	0.580	.209	2.775	0.348	0.416	0.837
Mean VLT-NF (cm)	136	2.444	0.363	2.807	1.440	0.040	0.470	0.349	.208	1.678	0.020	0.413	0.048
TUG completed	117	1	0	1	0.44	0.046	.498	0.262	.224	1.170	-1.965	0.444	-4.426
TUG time (s)	117	989.75	9.25	999	604.56	41.82	452.36	-0.284	.224	-1.268	-1.926	0.444	-4.338
Best HGS (Kg)	133	46.0	0.0	46.0	17.06	0.81	9.32	0.120	.210	0.571	0.249	0.417	0.597
Low grip strength (EWGSOP2)	119	1	0	1	0.98	0.01	0.13	-7.614	.222	34.297	56.931	0.440	129.388

Table 9.2 Mean EQ5D-Index, EQ5D-Health, GFI, RMI, BADL, Vastus lateralis thickness and TUG time values for patients with normal and low grip strength. Grip strength cut offs according to EWGSOP2 criteria. Statistical analysis; Independent samples t-test. *Levene's test for equality of variance not met therefore adjusted for equal variances not assumed.

	Grip strength (SD)				
	Normal	Low	t	df	P value
EQ5D-Index Day 7	0.315 (0.263)	0.156 (0.325)	3.068	128	0.003
EQ5D-Index Day 90	0.590 (0.372)	0.307 (0.346)	2.813*	59.983*	0.007*
EQ5D-Health Day 7	60.51 (20.71)	42.30 (21.50)	4.843	124	<.001
EQ5D-Health Day 90	66.19 (21.47)	51.38 (17.46)	3.109	72	0.003
GFI Pre-injury	6.83 (2.88)	8.00 (2.55)	-2.483	131	0.014
GFI Day 7	7.57 (2.28)	8.29 (2.20)	-1.836	129	0.069
GFI Day 90	6.35 (2.96)	8.40 (2.96)	-2.956	74	0.004
RMI Pre-injury	11.69 (2.76)	8.46 (3.35)	6.089*	128.231*	<.001*
RMI Day 7	3.75 (2.24)	2.30 (2.40)	3.574	129	<.001
RMI Day 90	9.07 (4.00)	5.90 (3.85)	3.419	74	0.001
BADL Pre-injury	95.00 (8.970)	74.71 (22.225)	6.960*	89.075*	<.001
BADL Day 7	59.62 (16.614)	36.14 (24.773)	6.380*	113.817*	<.001

	Grip strength (SD)				
	Normal	Low	t	df	P value
BADL Day 90	80.96 (21.810)	54.33 (32.582)	4.299*	45.609*	<.001
Mean VLT-F (cm)	1.90 (0.56)	1.63 (0.45)	2.936	129	0.004
Mean VLT-NF (cm)	1.60 (0.47)	1.30 (0.43)	3.945	131	<.001
TUG Time(s)	411.27 (435.03)	779.80 (395.75)	-4.709*	104.399*	<.001

Table 9.3 Mean difference EQ5D-Index and Health values from day 7 to day 90 for patients with normal and low grip strength. Statistical analysis with Paired t test.

	Index				Health			
Grip Strength	Mean Δ(SD)	t	df	P value	Mean Δ(SD)	t	df	P value
Normal	0.335 (0.337)	6.521	42	<.001	6.97 (28.80)	1.623	44	0.112
Low	0.167 (0.366)	2.544	30	0.016	4.44 (24.69)	0.936	26	0.358

Table 9.4 Mean EQ5D-Index and Health values for patients able and unable to complete TUG test. Statistical analysis with Independent samples t-test. *Levene's test for equality of variance not met therefore adjusted for equal variances not assumed.

	Able to complete TUG test				
	Yes	No	t	df	P value
EQ5D-Index					
Day 7 (SD)	0.341 (0.307)	0.118 (0.259)	-4.176	111	<.001
Day 90 (SD)	0.583 (0.250)	0.327 (0.388)	-3.037*	49.513*	0.004*
EQ5D-Health					
Day 7 (SD)	57.86 (19.79)	43.63 (22.56)	-3.458	107	<.001
Day 90 (SD)	63.97 (18.83)	49.71 (19.78)	-2.861	58	0.006

Table 9.5 Mean difference EQ5D-Index and Health values from day 7 to day 90 for patients able and unable to complete TUG test. Statistical analysis with Paired t test.

	Index				Health			
	Mean Δ(SD)	t	df	P value	Mean Δ(SD)	t	df	P value
Completed TUG								
Yes	0.254 (0.339)	4.095	29	<.001	7.433 (26.83)	1.518	29	0.140
No	0.246 (0.412)	3.266	29	0.003	2.554 (30.45)	0.444	27	0.661

Table 9.6 Mean difference in GFI, RMI, and BADL within normal and low grip strength groups, comparing day 7 with pre-injury, day 90 with pre-injury, and day 90 with day 7. Statistical analysis with paired t test.

Grip strength		GFI Mean Δ (SD)	t value	df	P value	RMI Mean Δ (SD)	t value	df	P value	BADL Mean Δ (SD)	t value	df	P value
Normal	Day 7 & Pre-injury	0.738 (3.007)	1.980	64	0.052	-7.938 (2.882)	-22.204	64	<.001	-35.385 (17.574)	-16.233	64	<.001
	Day 90 & Pre-injury	-0.261 (2.113)	-0.837	45	0.407	-2.804 (2.833)	-6.713	45	<.001	-14.787 (19.725)	-5.139	46	<.001
	Day 90 & Day 7	-1.391 (3.180)	-2.968	45	0.005	5.239 (3.761)	9.449	45	<.001	22.234 (21.964)	6.940	46	<.001
Low	Day 7 & Pre-injury	0.409 (2.992)	1.111	65	0.271	-6.197 (3.254)	-15.469	65	<.001	-38.712 (20.872)	-15.068	65	<.001
	Day 90 & Pre-injury	0.133 (2.909)	0.251	29	0.804	-3.333 (4.147)	-4.403	29	<.001	-24.167 (25.666)	-5.157	29	<.001
	Day 90 & Day 7	-0.233 (2.542)	-0.503	29	0.619	3.400 (3.125)	5.959	29	<.001	10.500 (25.742)	2.234	29	0.033

Table 9.7. Mean RMI, and BADL for patients able and unable to complete TUG test. Note. Independent samples t-test. *Levene's test for equality of variance not met therefore adjusted for equal variances not assumed.

	Able to complete TUG test (SD)				
	Yes	No	t	df	P value
RMI Pre-Injury	11.08 (2.883)	8.64 (3.515)	-4.126*	114.563*	<.001*
RMI Day 7	3.94 (2.368)	1.98 (1.856)	-4.944	112	<.001
RMI Day 90	9.00 (3.521)	5.61 (4.014)	-3.532	60	<.001
BADL Pre-injury	94.02 (9.382)	76.51 (22.846)	-5.932*	90.842*	<.001*
BADL Day 7	60.80 (15.365)	33.44 (21.323)	-7.957*	111.339*	<.001*
BADL Day 90	82.03 (16.405)	52.58 (33.012)	-4.462*	43.653*	<.001

Table 9.8 Mean difference RMI and BADL for patients able and unable to complete TUG test comparing day 7 with pre-injury, day 90 with pre-injury, and day 90 with day 7. Statistical analysis with paired t test.

Able to complete TUG		RMI				BADL			
		Mean Δ (SD)	t value	df	P value	Mean Δ (SD)	t value	df	P value
Yes	Day 7 & Pre-injury	-7.160 (3.158)	-16.031	49	<.001	-33.200 (16.560)	-14.176	49	<.001
	Day 90 & Pre-injury	-2.645 (3.147)	-4.680	30	<.001	-13.750 (16.848)	-4.617	31	<.001
	Day 90 & Day 7	4.742 (3.336)	7.913	30	<.001	19.219 (20.165)	5.391	31	<.001
No	Day 7 & Pre-injury	-6.703 (3.346)	-16.026	63	<.001	-42.344 (20.644)	-16.409	63	<.001
	Day 90 & Pre-injury	-3.742 (3.949)	-5.275	30	<.001	-28.548 (27.270)	-5.829	30	<.001
	Day 90 & Day 7	3.645 (3.937)	5.155	30	<.001	13.710 (29.579)	2.581	30	0.015

Table 9.9 Crosstabulation ability to complete TUG test and low grip strength.

		Low grip strength EQGSOP2		
		No	Yes	Total
Able to complete TUG	No	18	48	66
	Yes	34	15	49
Total		52	63	115

Table 9.10 Chi-Squared test [a]0 cells (0.0%) have expected count less than 5. The minimum expected count is 22.16. [b]Computed only for a 2x2 table.

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig (1-sided)
Pearsons Chi-Square	20.136[a]	1	<.001		
Continuity Correction [b]	18.471	1	<.001		
Likelihood ratio	20.660	1	<.001		
Fisher's Exact Test				<.001	<.001
Linear by Linear Association	19.961	1	<.001		
N of valid cases	115				

9.1.2.9 Figures of results

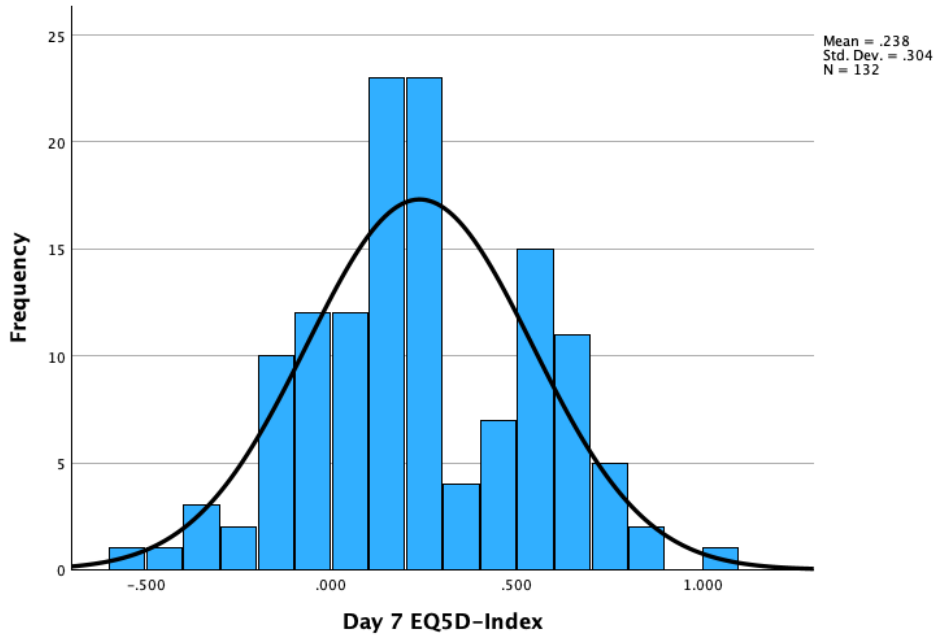


Figure 9.1. *Histogram of frequency and day 7 EuroQol-5D-Index value*

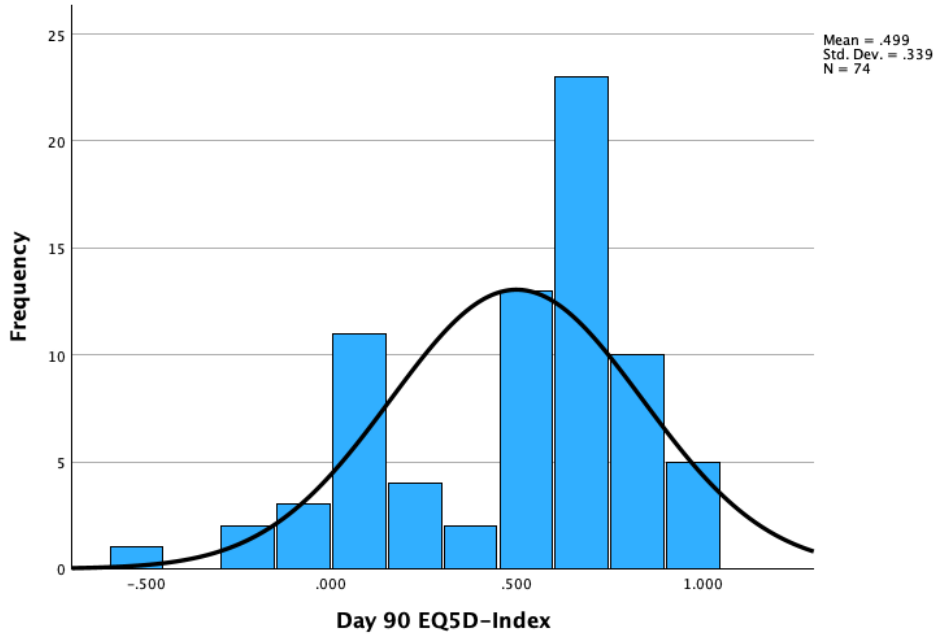


Figure 9.2. *Histogram of frequency and day 90 EuroQol-5D -Index value*

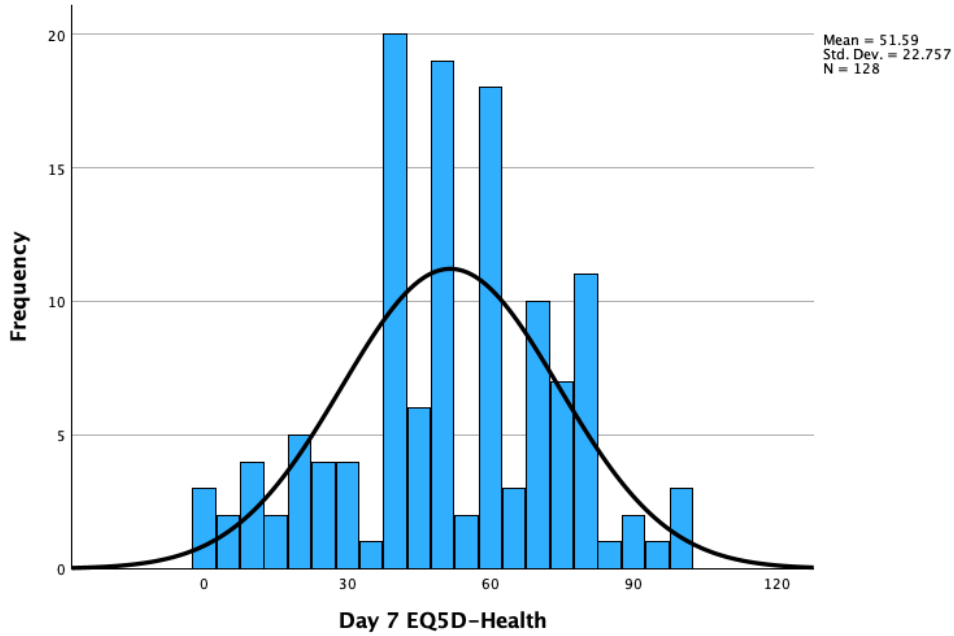


Figure 9.3. *Histogram of frequency and day 7 EuroQol-5D -Health value*

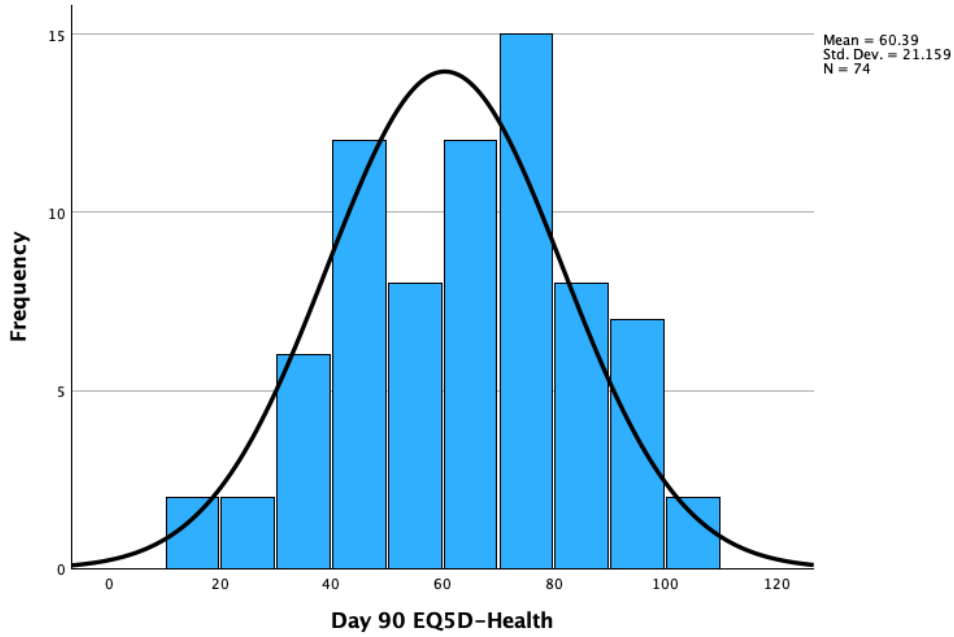


Figure 9.4. *Histogram of frequency and day 90 EuroQol-5D -Health value*

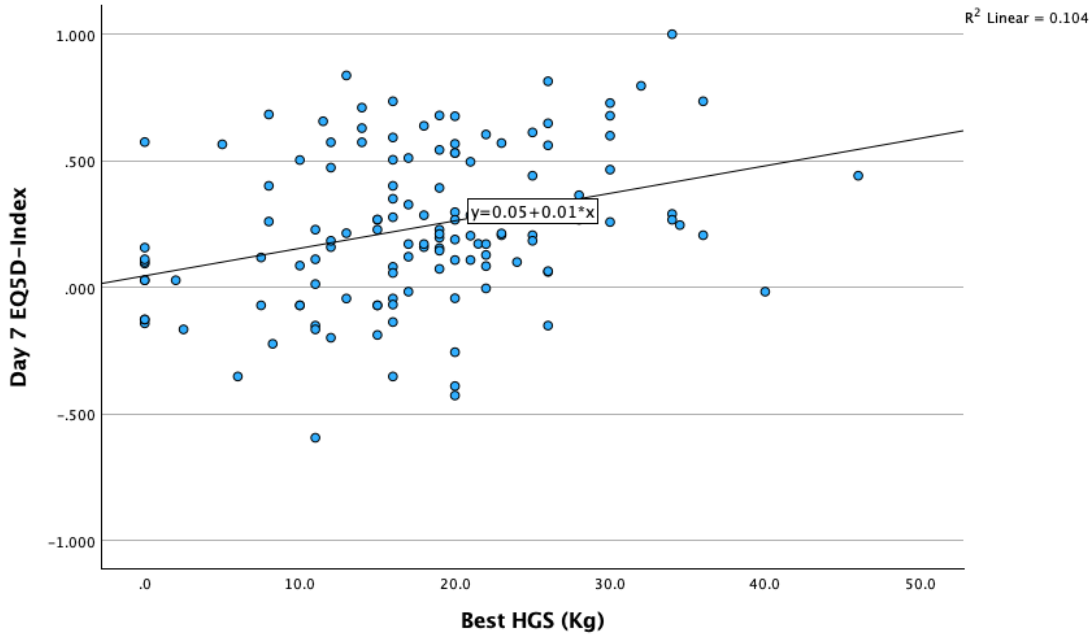


Figure 9.5. *Scatter plot day 7 EuroQol-5D -Index vs best hand grip strength (Kg)*

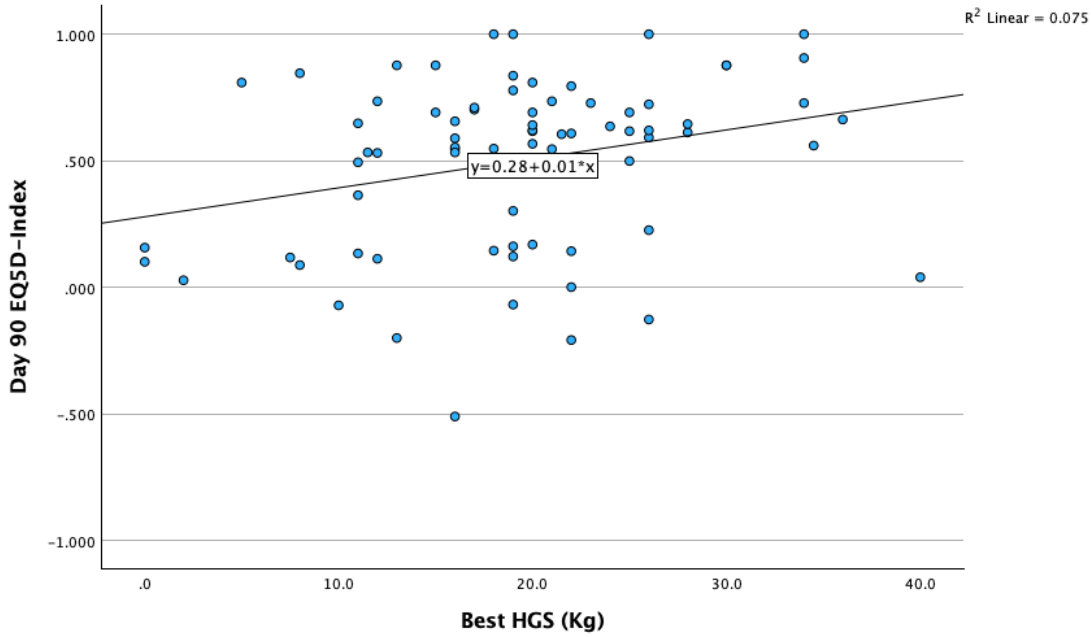


Figure 9.6. *Scatter plot day 90 EuroQol-5D -Index vs best hand grip strength (Kg)*

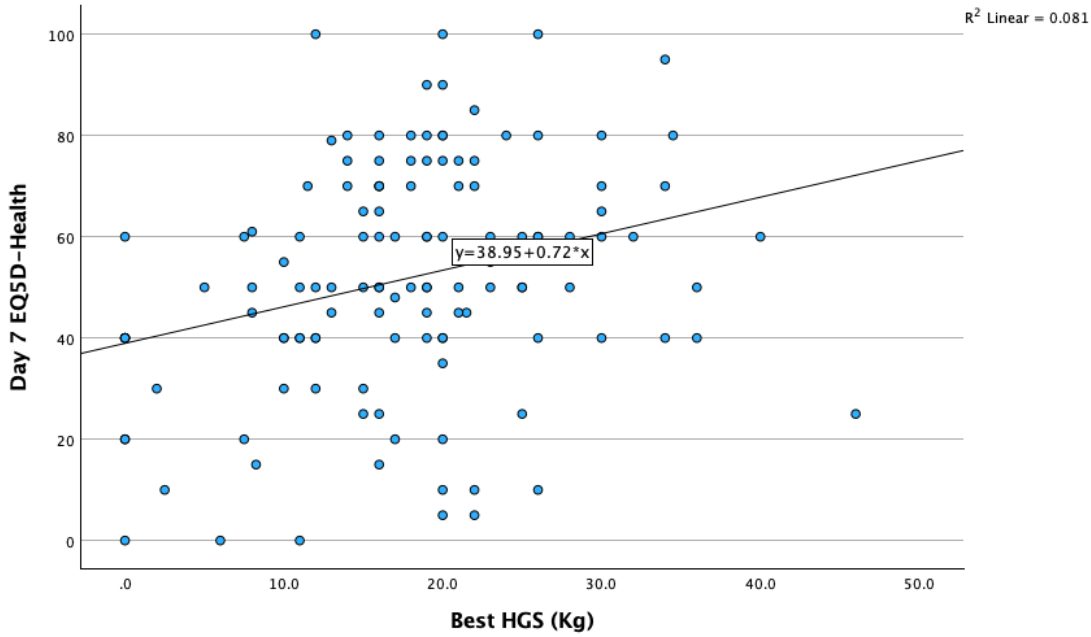


Figure 9.7. *Scatter plot day 7 EuroQol-5D -Health vs best hand grip strength (Kg)*

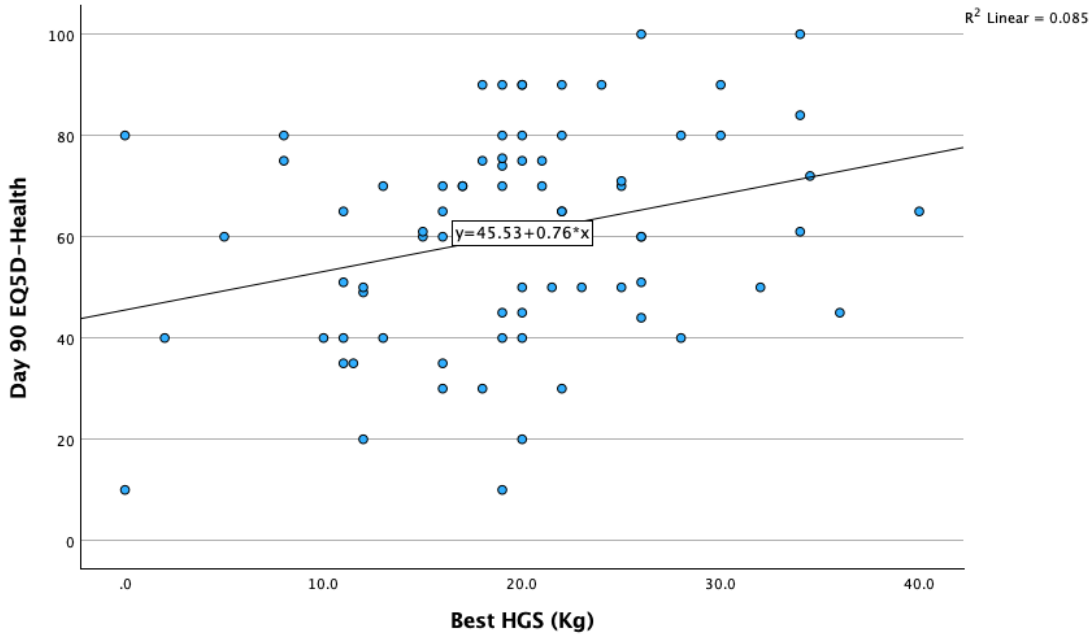


Figure 9.8. *Scatter plot day 90 EuroQol-5D -Health vs best hand grip strength (Kg)*

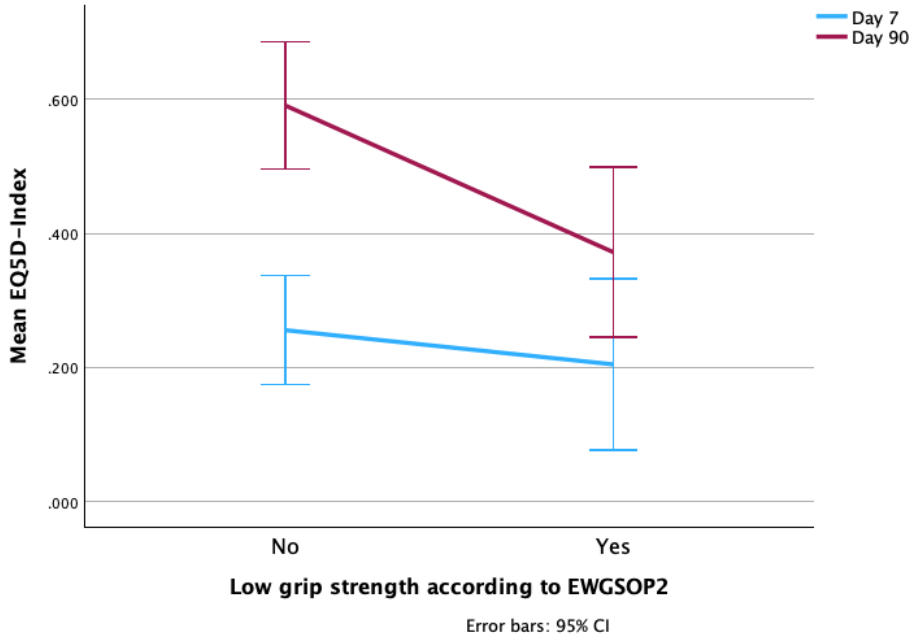


Figure 9.9. *Mean EuroQol-5D -Index and grip strength according to EWGSOP2 criteria*

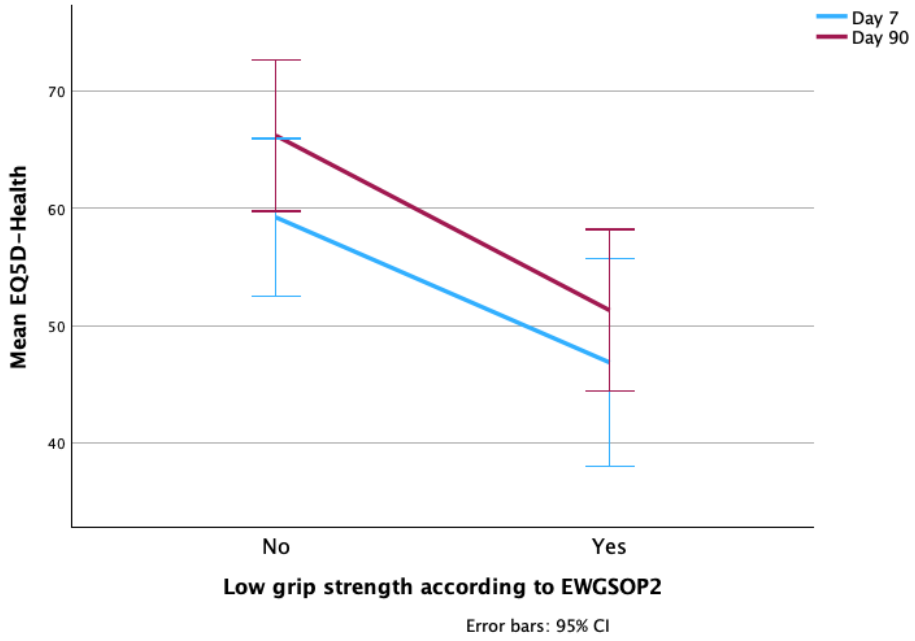


Figure 9.10. *Mean EuroQol-5D -Health and grip strength according to EWGSOP2 criteria*

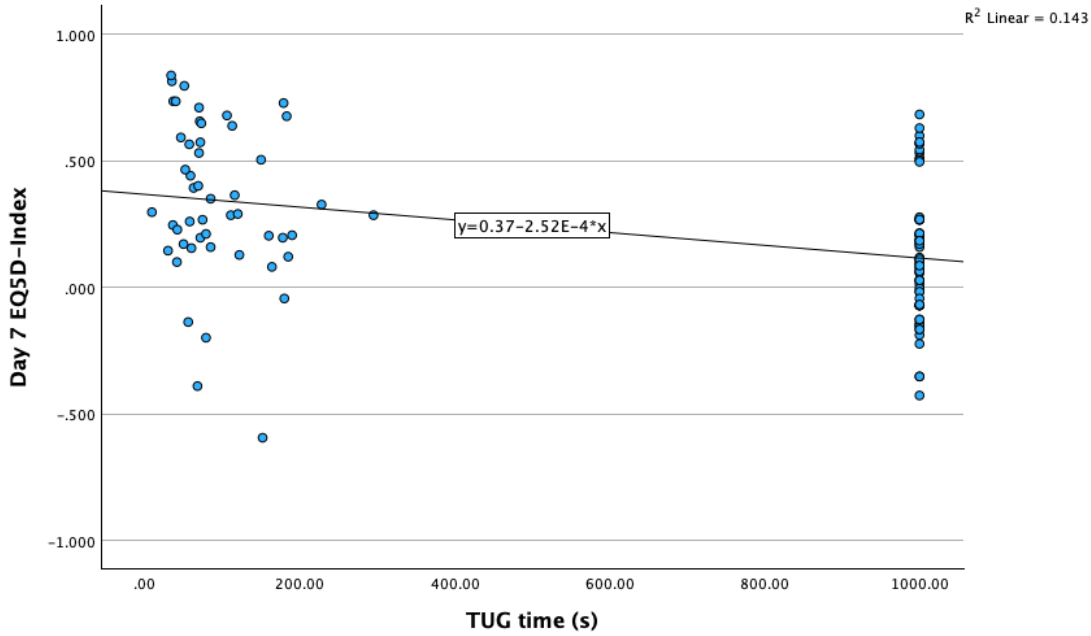


Figure 9.11. *Scatter plot day 7 EuroQol-5D -Index vs Timed up and go test time (s)*

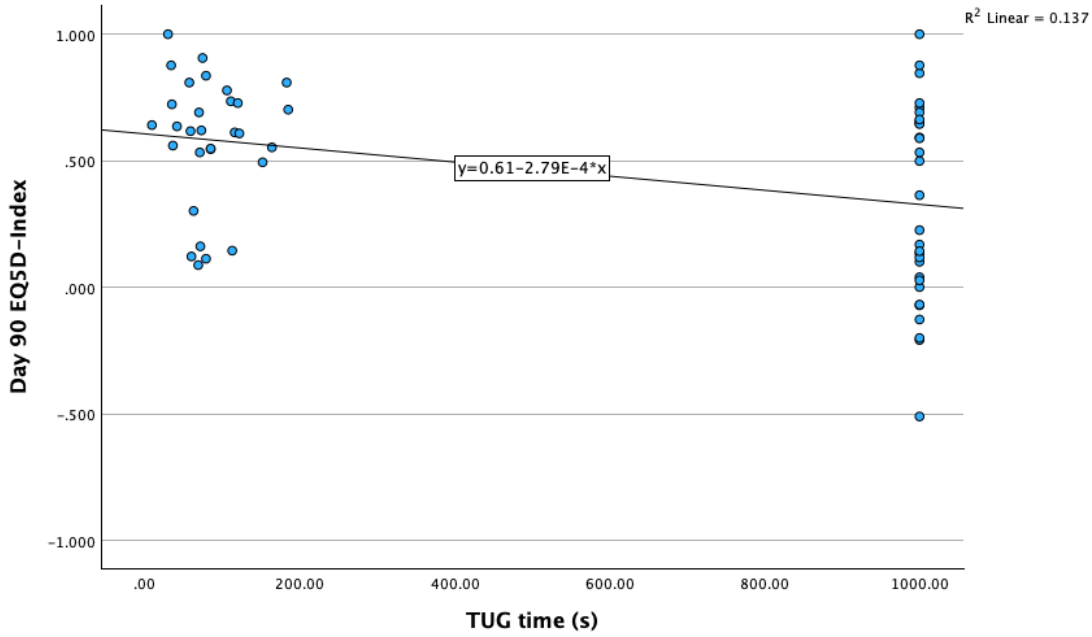


Figure 9.12. *Scatter plot day 90 EuroQol-5D -Index vs Timed up and go test time (s)*

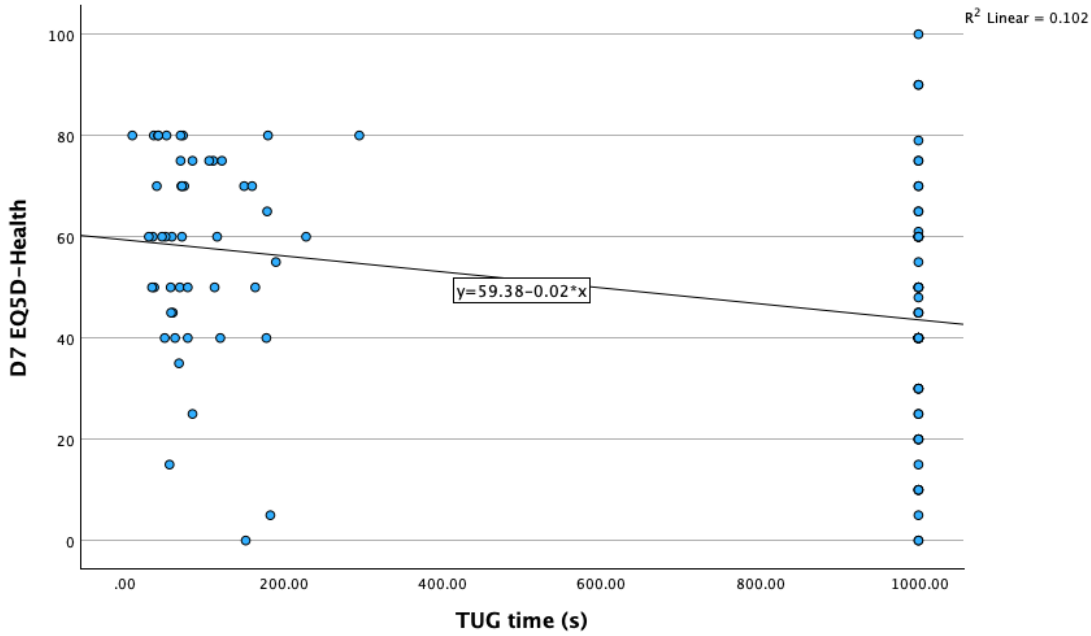


Figure 9.13. *Scatter plot day 7 EuroQol-5D -Health vs Times up and go test time (s)*

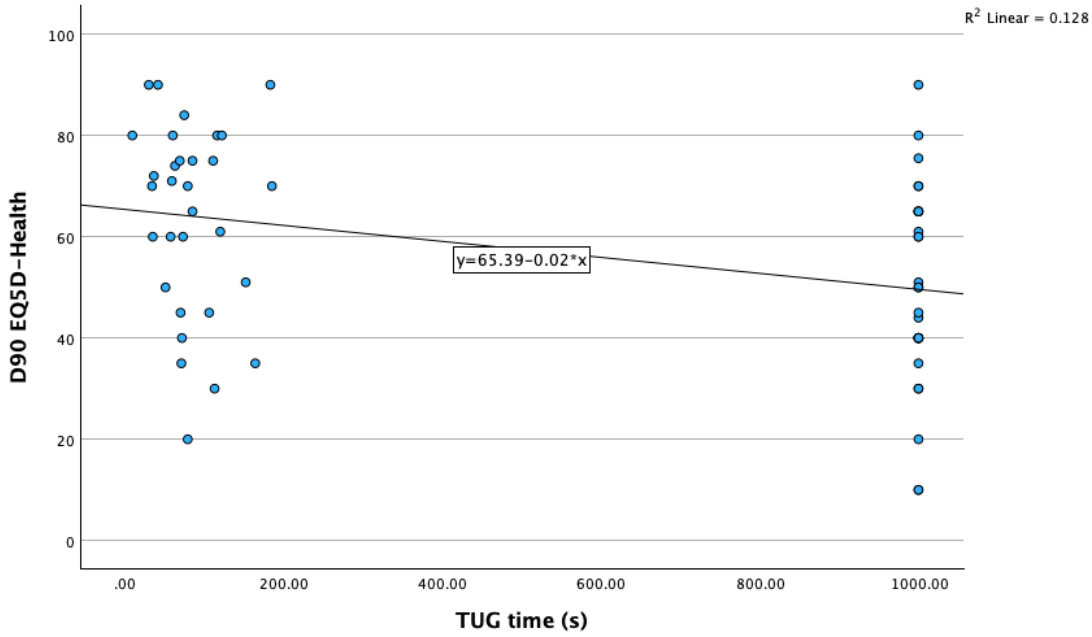


Figure 9.14. *Scatter plot day 90 EuroQol-5D -Health vs Timed up and go test time (s)*

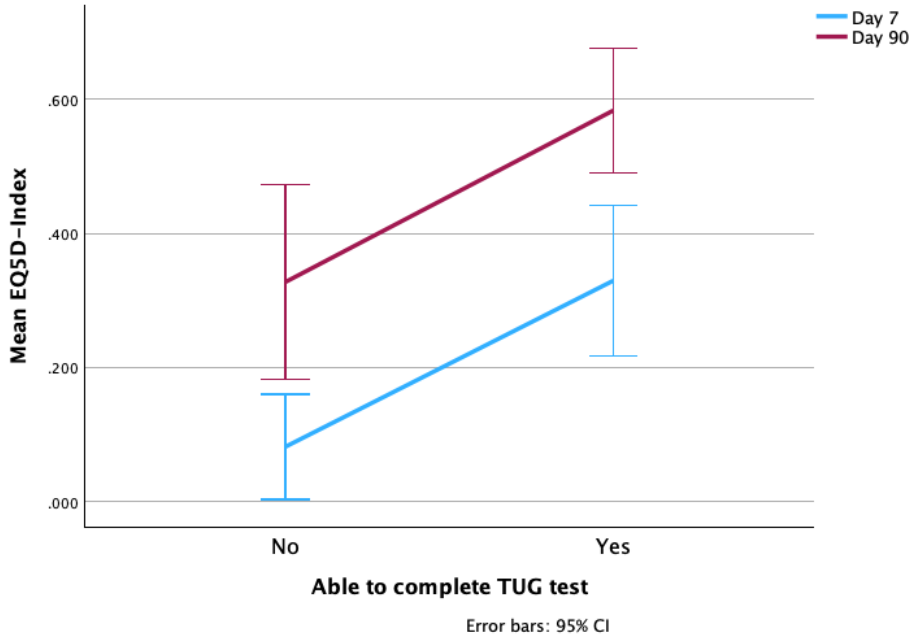


Figure 9.15. *Mean EuroQol-5D -Index and the ability to complete timed up and go test.*

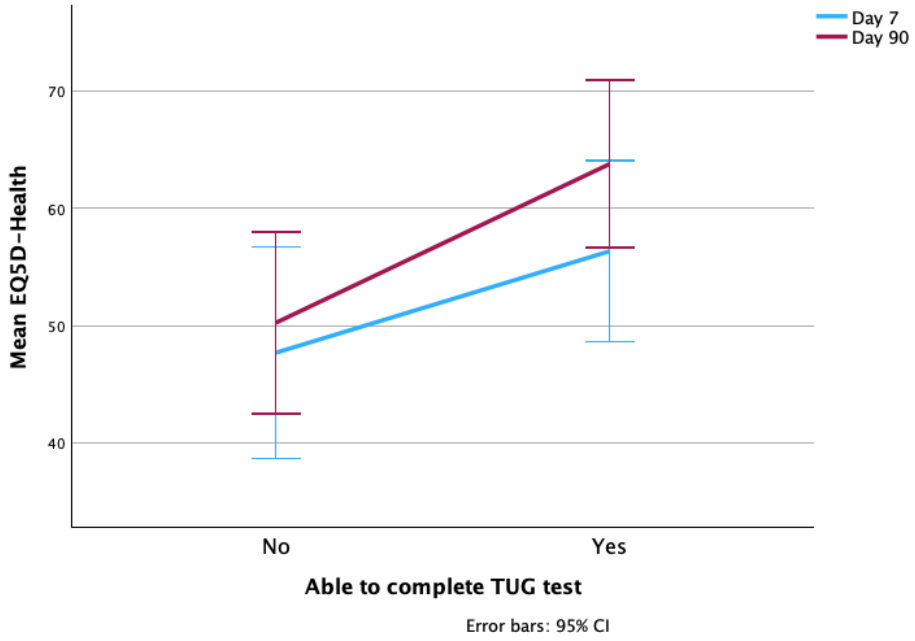


Figure 9.16. *Mean EuroQol-5D -Health and the ability to complete Timed up and go test.*

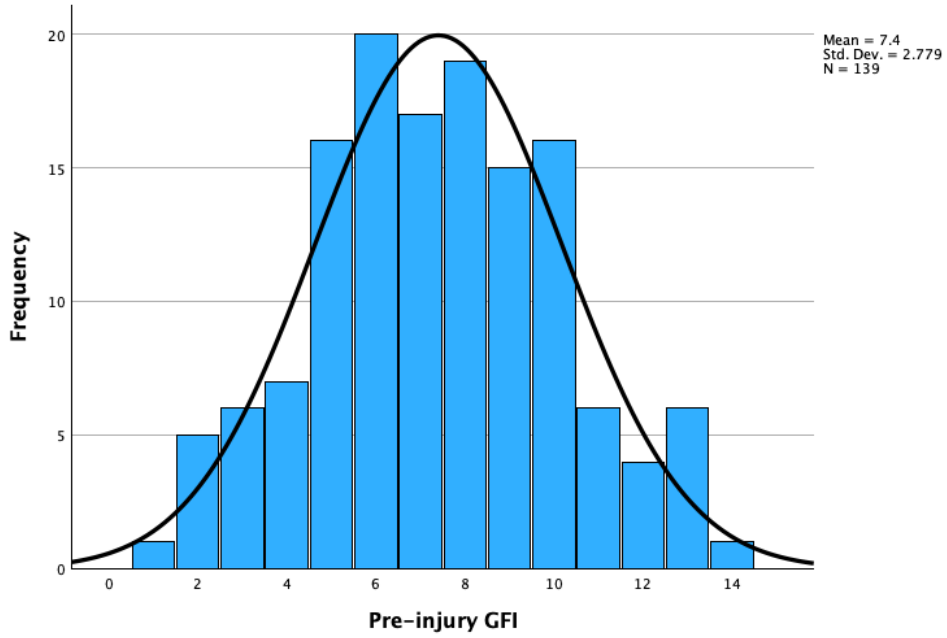


Figure 9.17. *Histogram of frequency vs pre-injury Groningen frailty index*

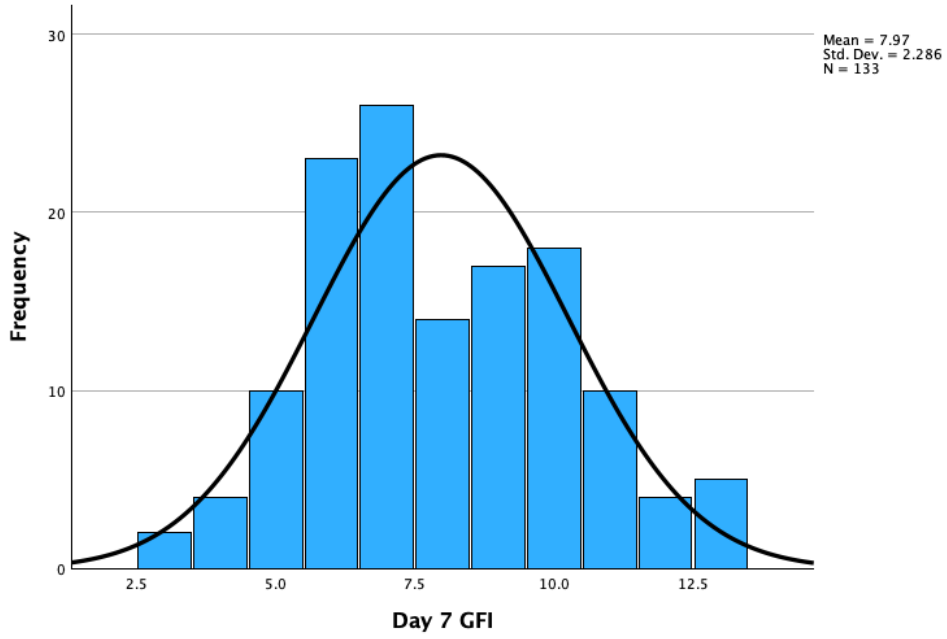


Figure 9.18. *Histogram of frequency vs day 7 Groningen frailty index*

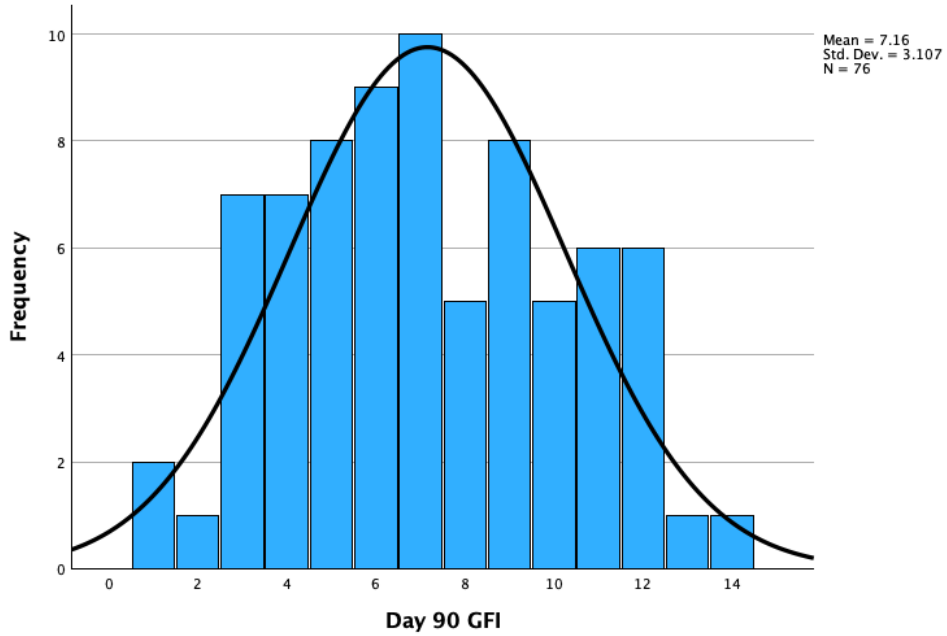


Figure 9.19. *Histogram of frequency vs day 90 Groningen frailty index*

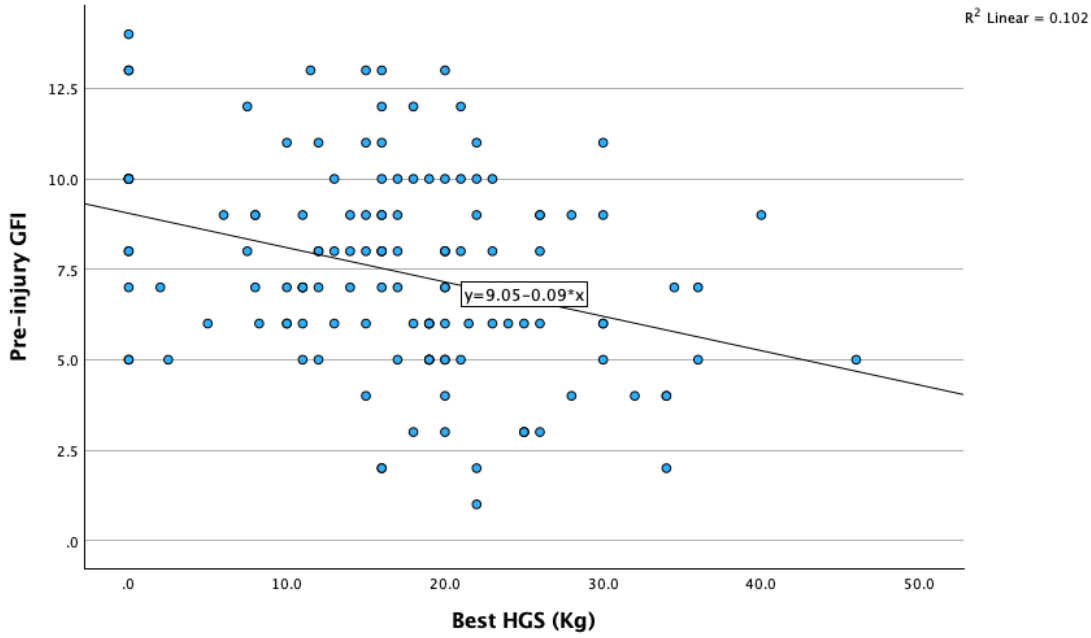


Figure 9.20. *Pre-Injury Groningen frailty index vs best hand grip strength (Kg)*

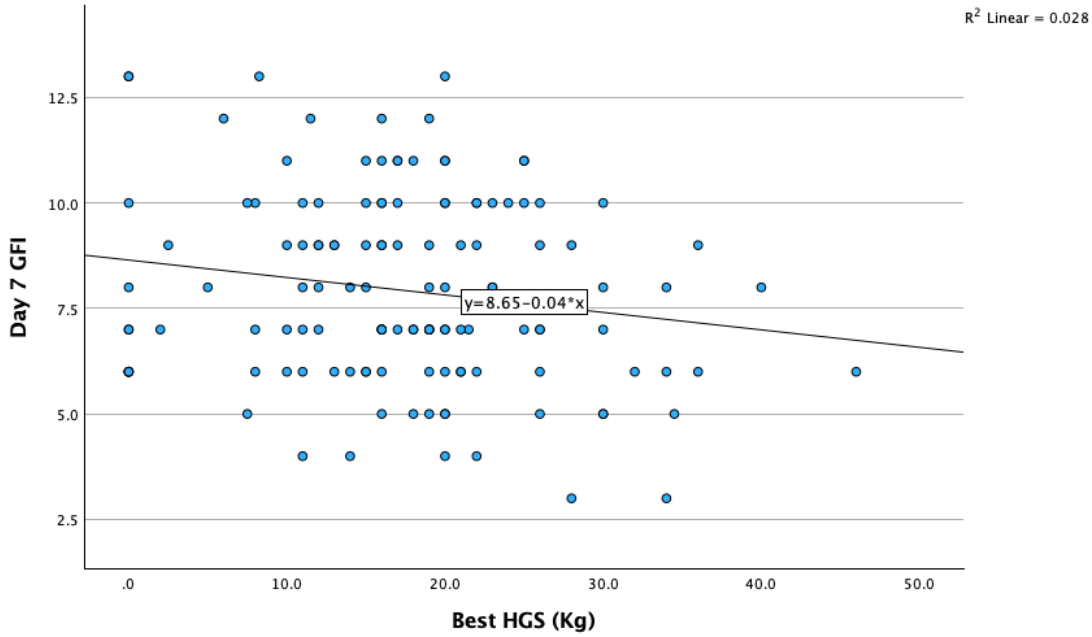


Figure 9.21. *Day 7 Groningen frailty index vs best hand grip strength (Kg)*

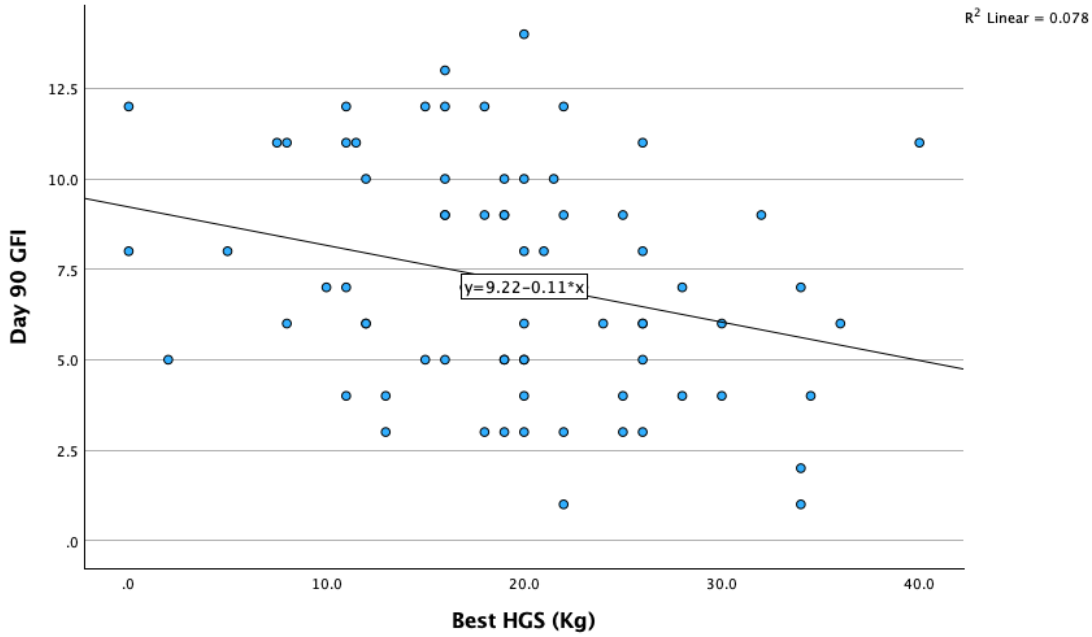


Figure 9.22. *Day 90 Groningen frailty index vs best hand grip strength (Kg)*

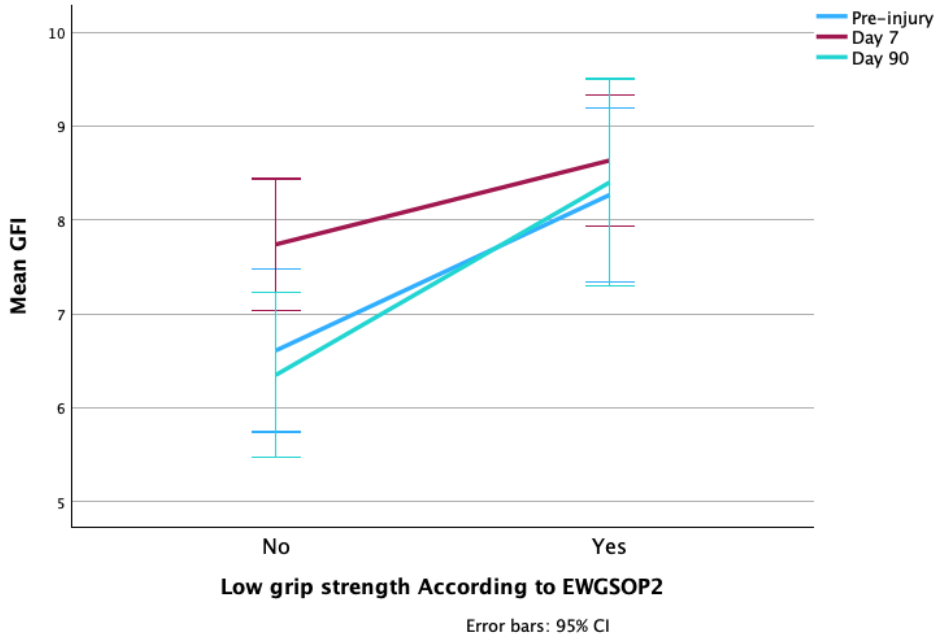


Figure 9.23. *Mean Groningen frailty index (pre-injury, day 7 and day 90 vs grip strength according to EWGSOP2 cut-offs*

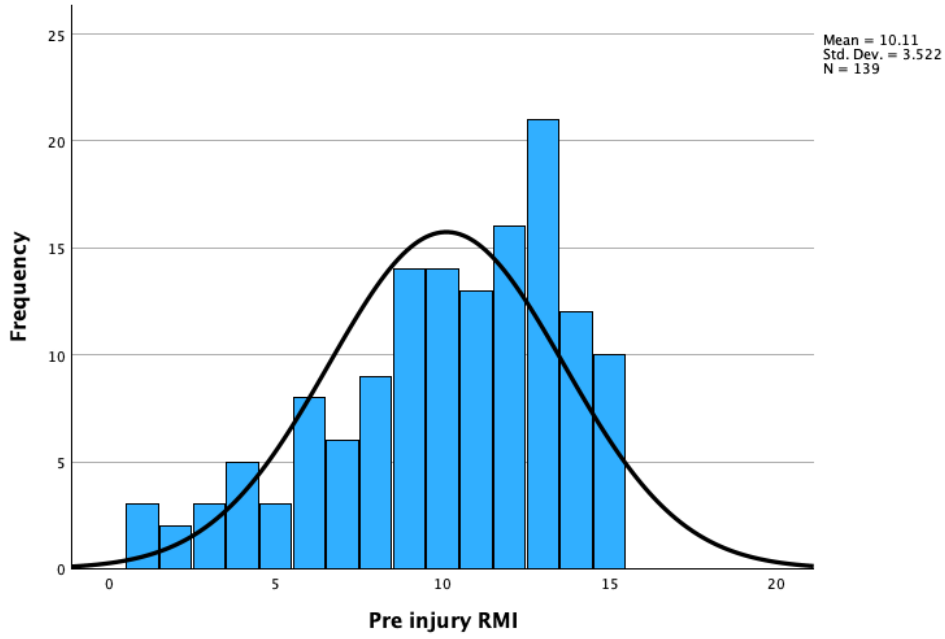


Figure 9.24. *Histogram of frequency vs pre-injury Rivermead mobility index*

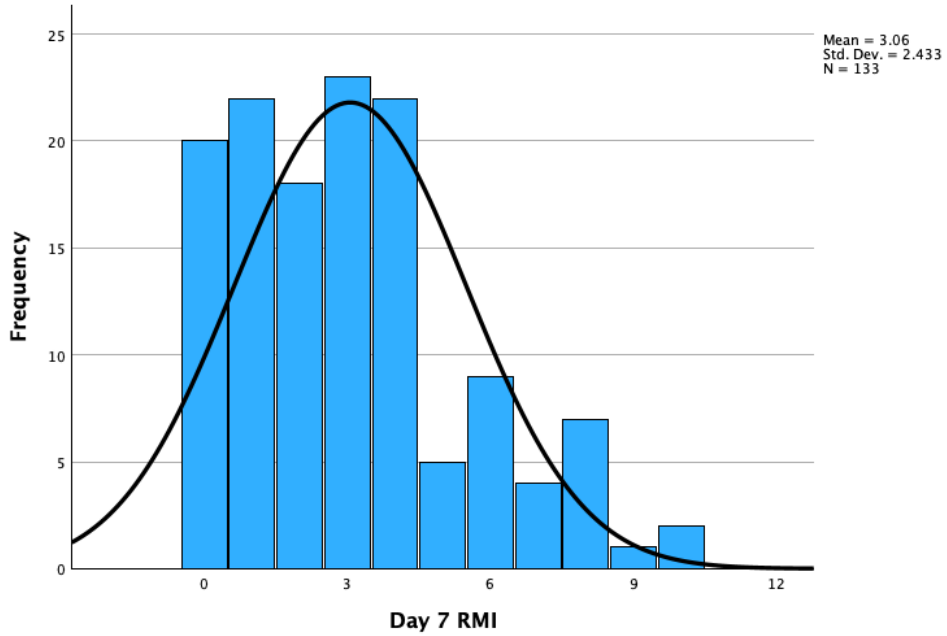


Figure 9.25. *Histogram of frequency vs day 7 Rivermead mobility index*

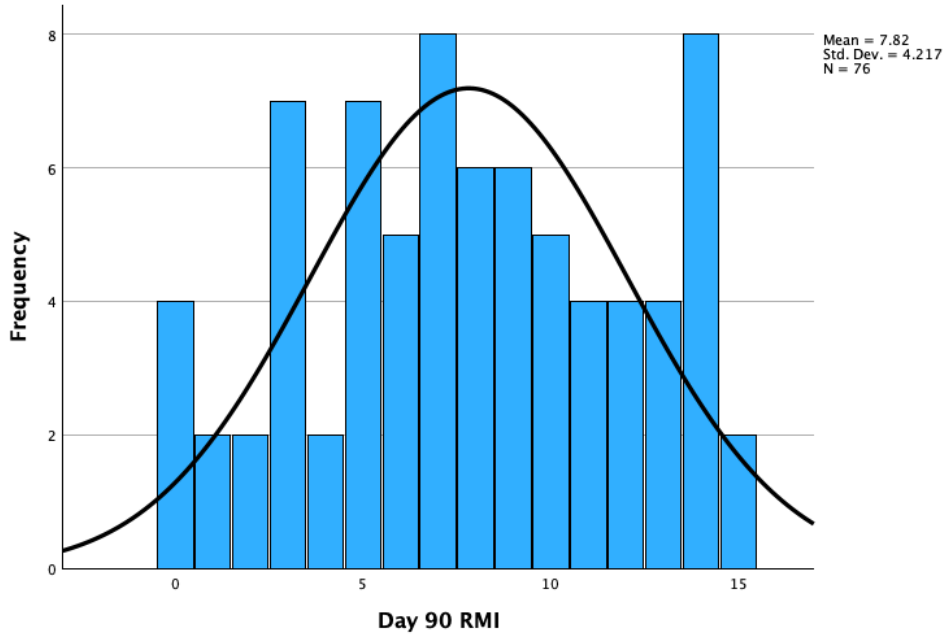


Figure 9.26. *Histogram of frequency vs day 90 Rivermead mobility index*

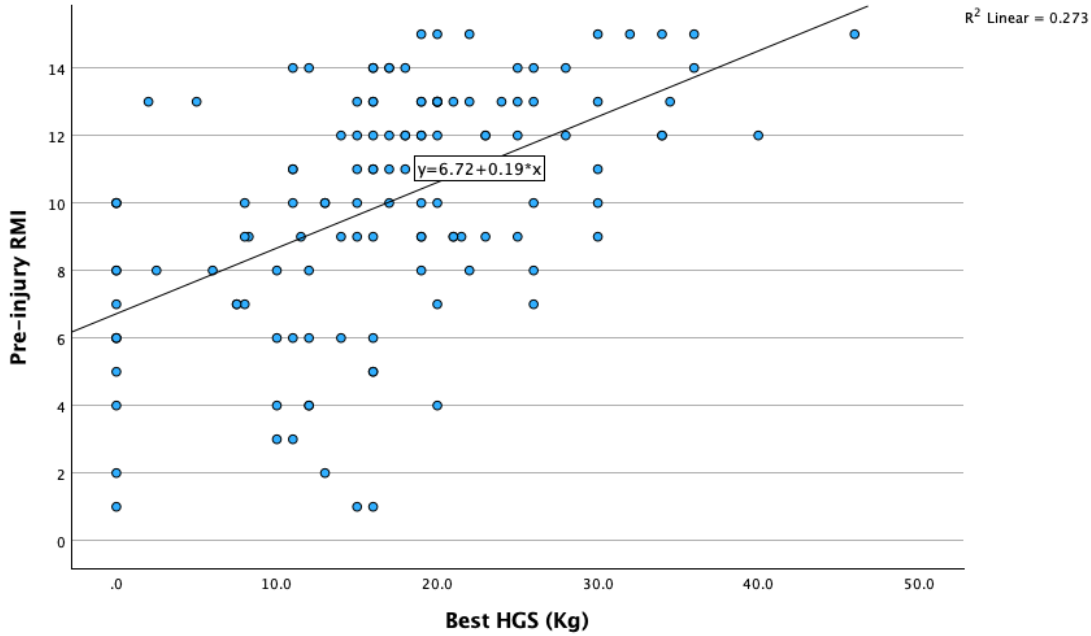


Figure 9.27. Scatter plot of *Pre-injury Rivermead Mobility Index and Hand grip strength (Kg)*

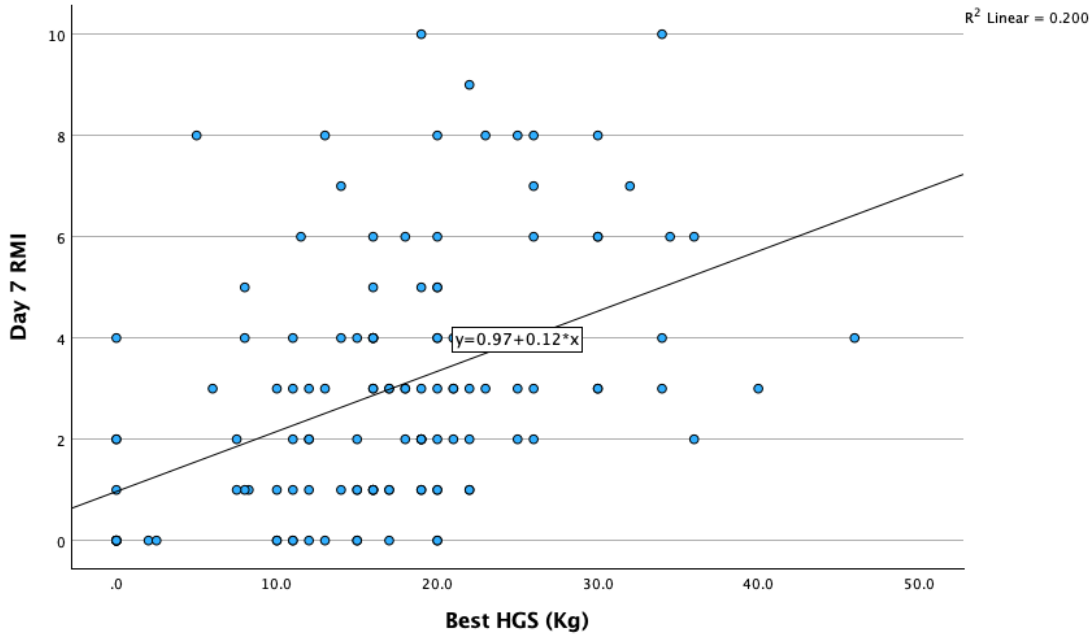


Figure 9.28. Scatter plot of *Day 7 Rivermead Mobility Index and Hand grip strength (Kg)*

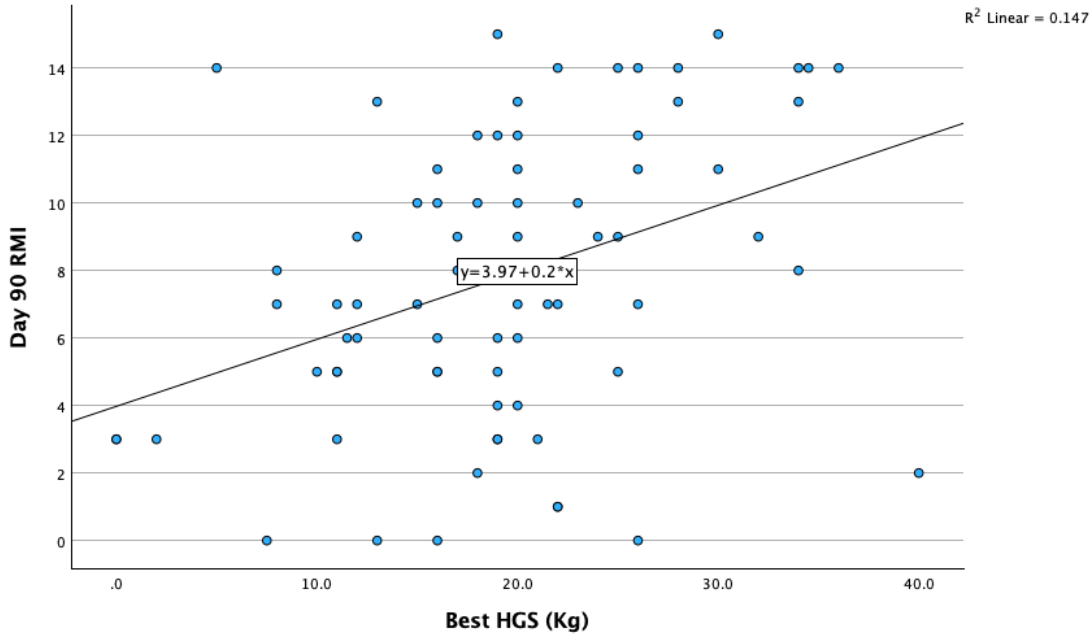


Figure 9.29. Scatter plot of *Day 90 Rivermead Mobility Index and Hand Grip Strength (Kg)*

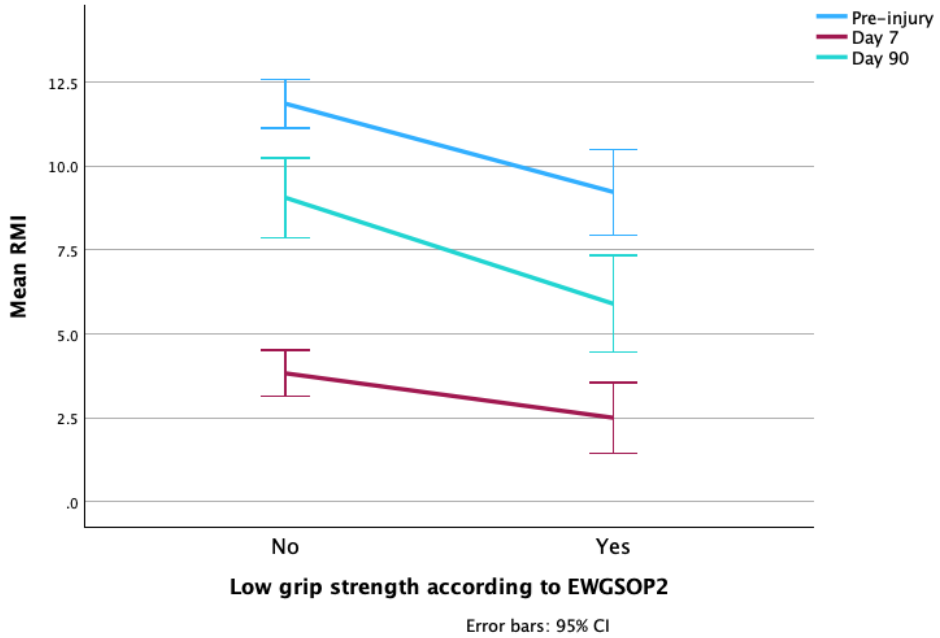


Figure 9.30. *Mean Rivermead mobility index and grip strength according to EWGSOP2 cut-offs*

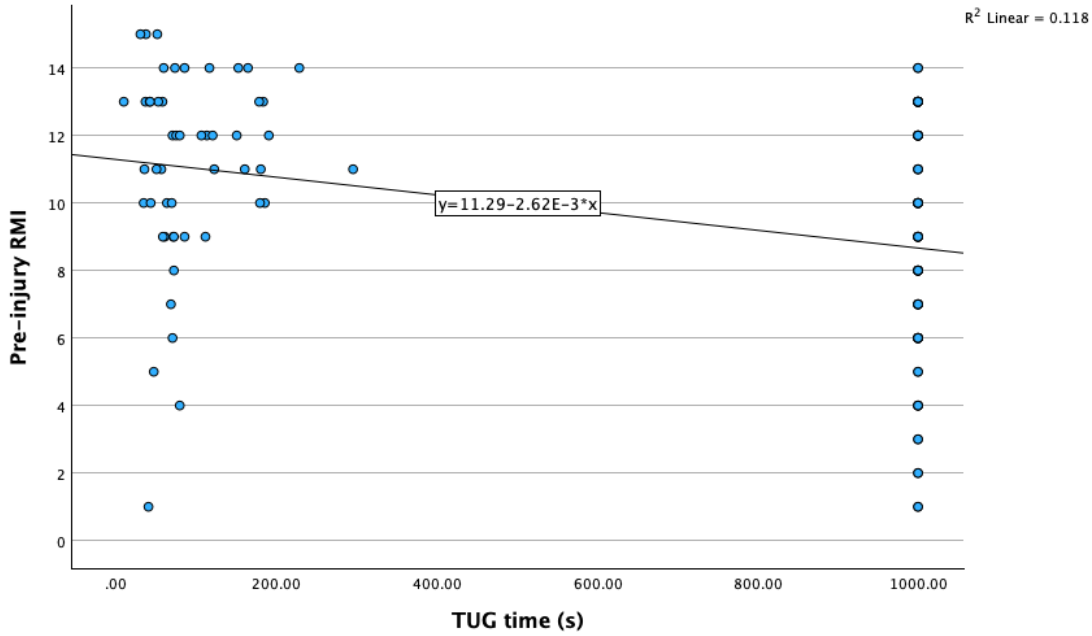


Figure 9.31. *Pre-injury Rivermead mobility index and Timed up and go test time*

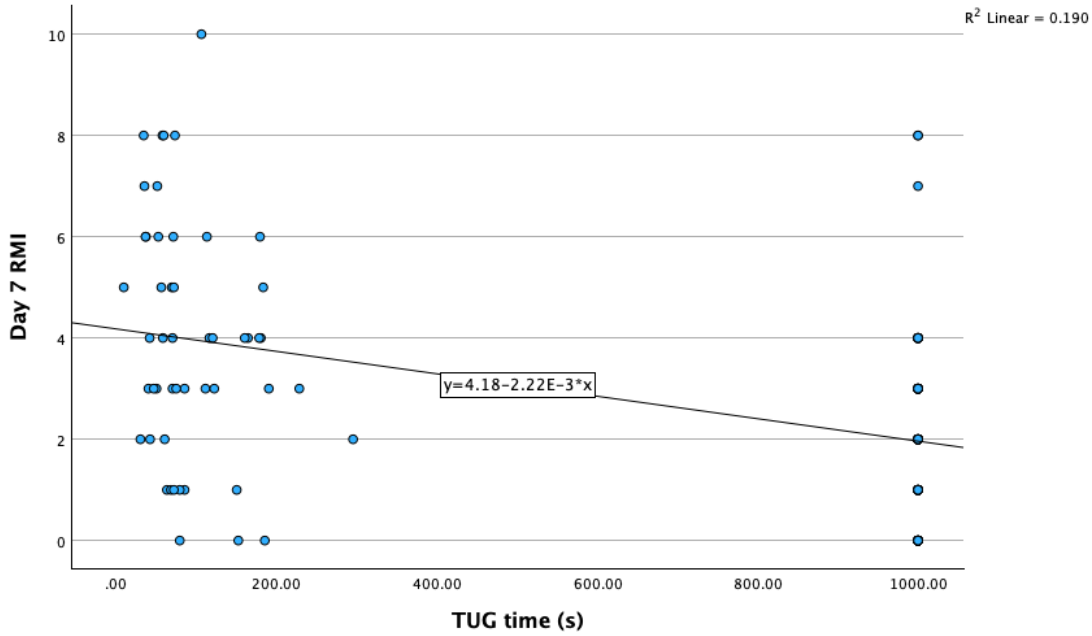


Figure 9.32. *Day 7 Rivermead mobility index and Timed up and go test time*

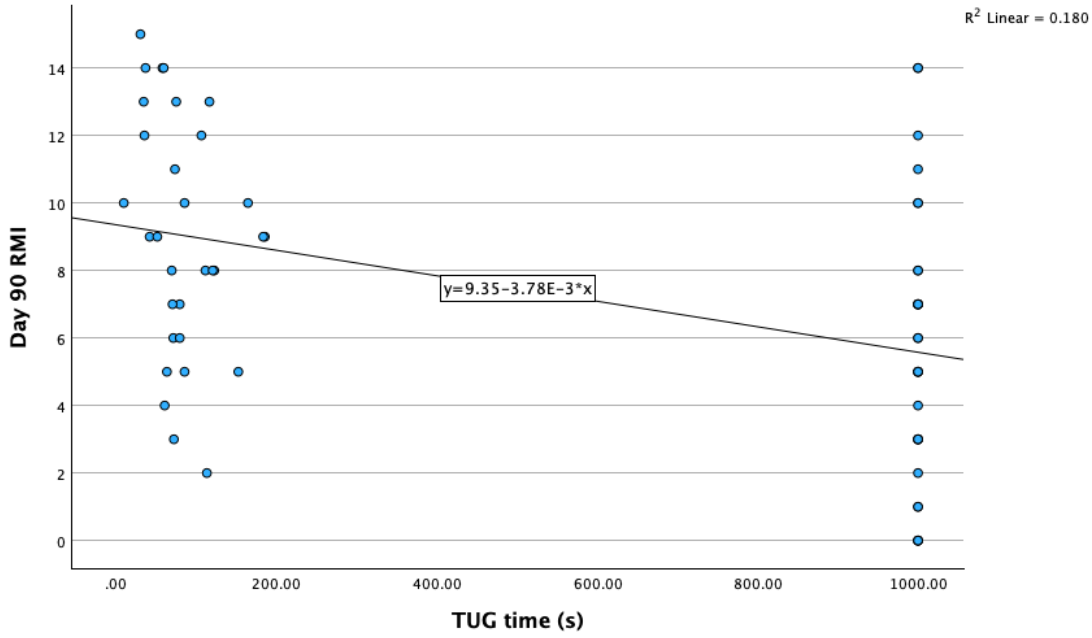


Figure 9.33. *Day 90 Rivermead mobility index and Timed up and go test time*

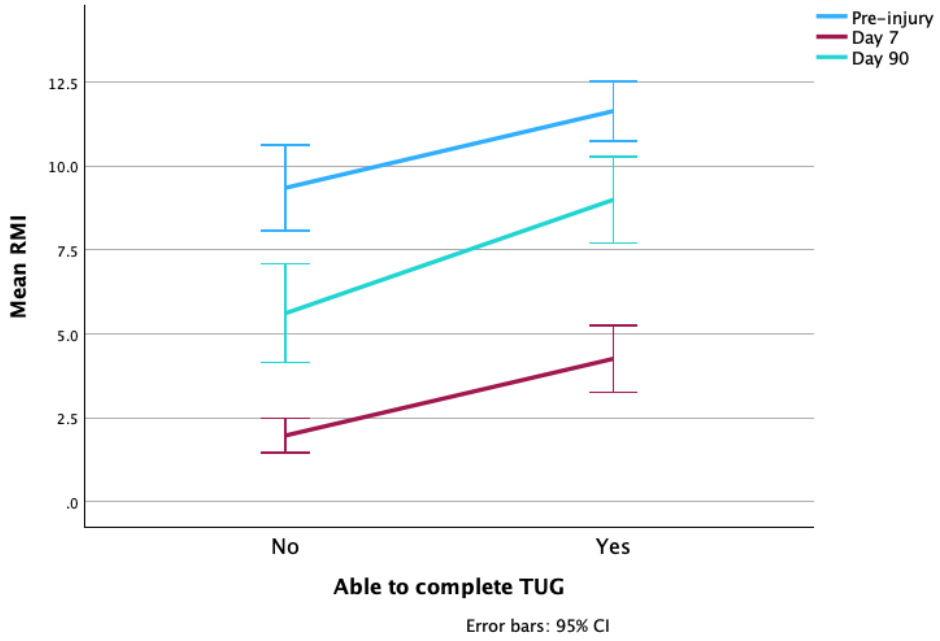


Figure 9.34. *Mean Rivermead mobility index and ability to complete Timed up and go test*

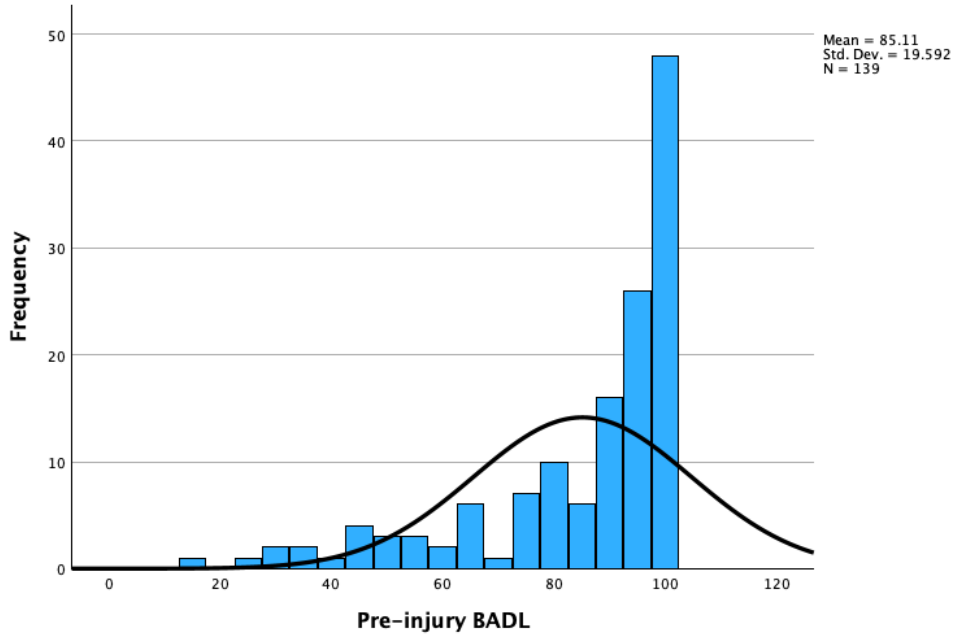


Figure 9.35. *Histogram of frequency vs pre-injury Barthels activity of daily living*

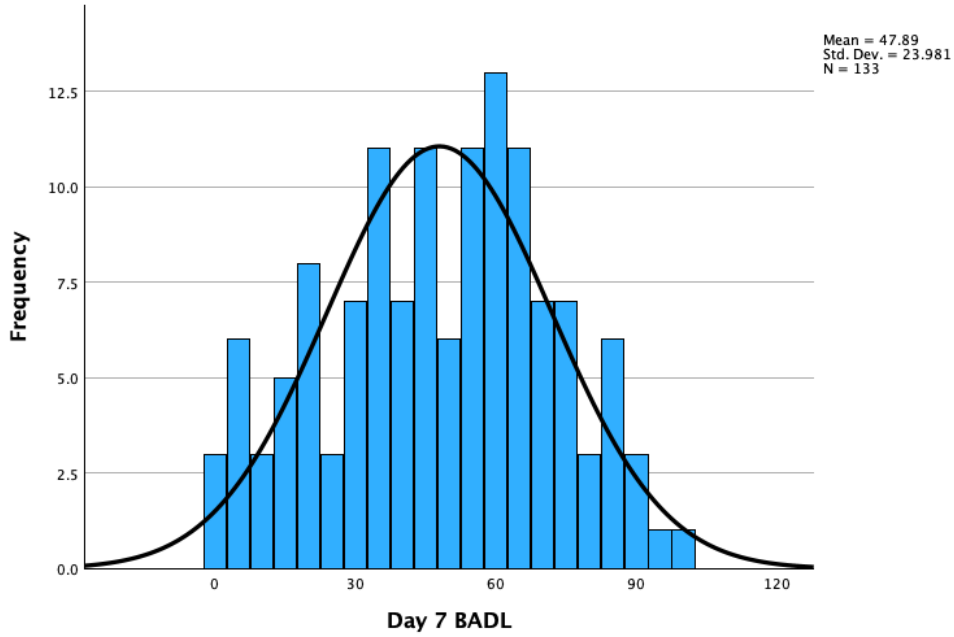


Figure 9.36. *Histogram of frequency vs day 7 Barthels activity of daily living*

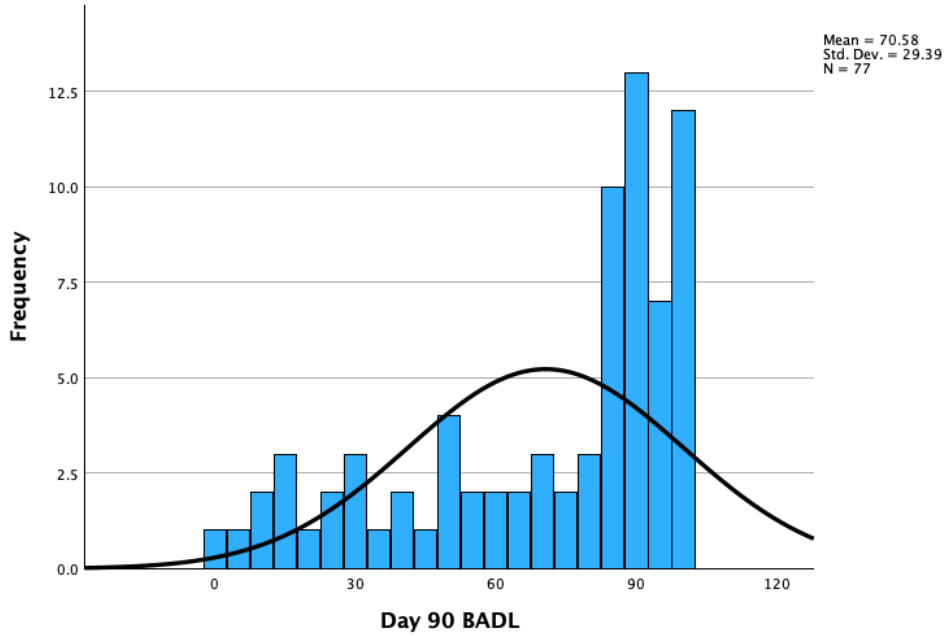


Figure 9.37. *Histogram of frequency vs day 90 Barthels activity of daily living*

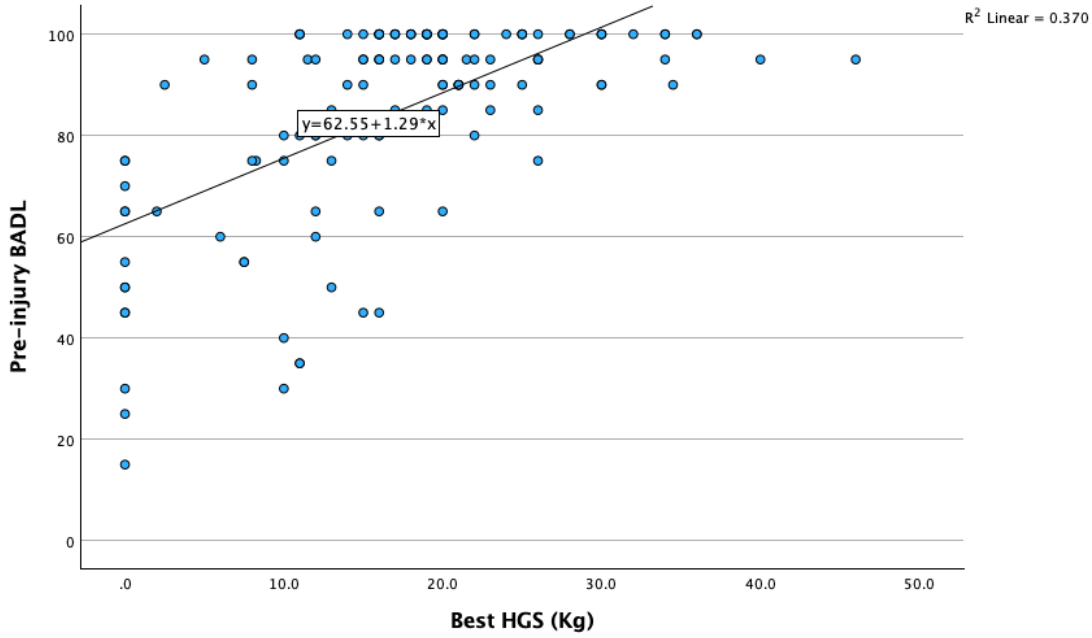


Figure 9.38. *Pre-injury Barthels activity of daily living and best hand grip strength (Kg)*

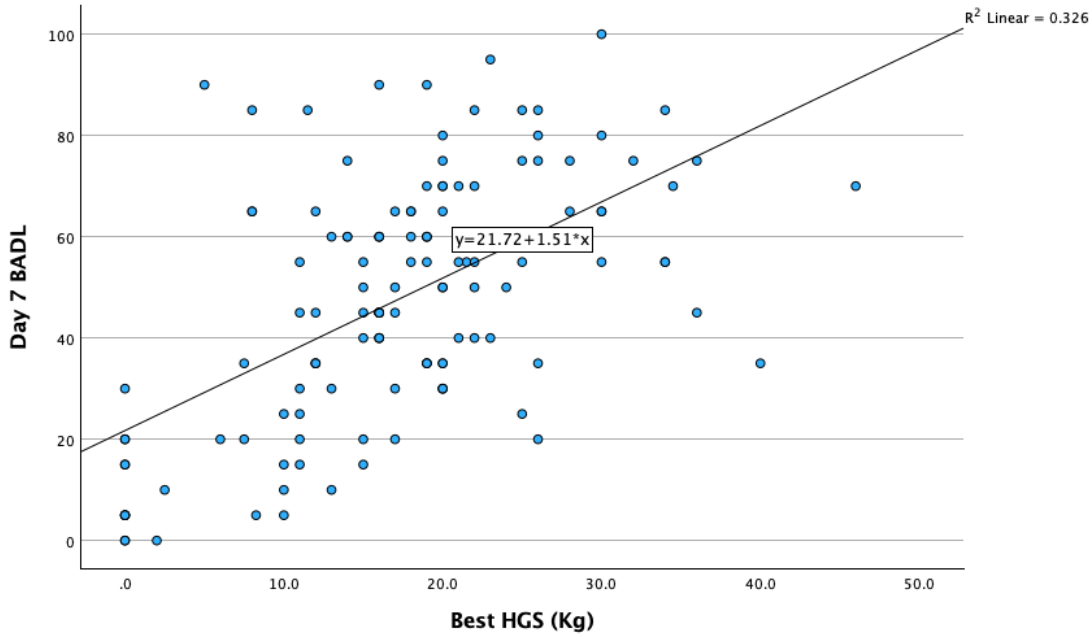


Figure 9.39. Scatter plot *Day 7 Barthels activity of daily living and best hand grip strength(Kg)*

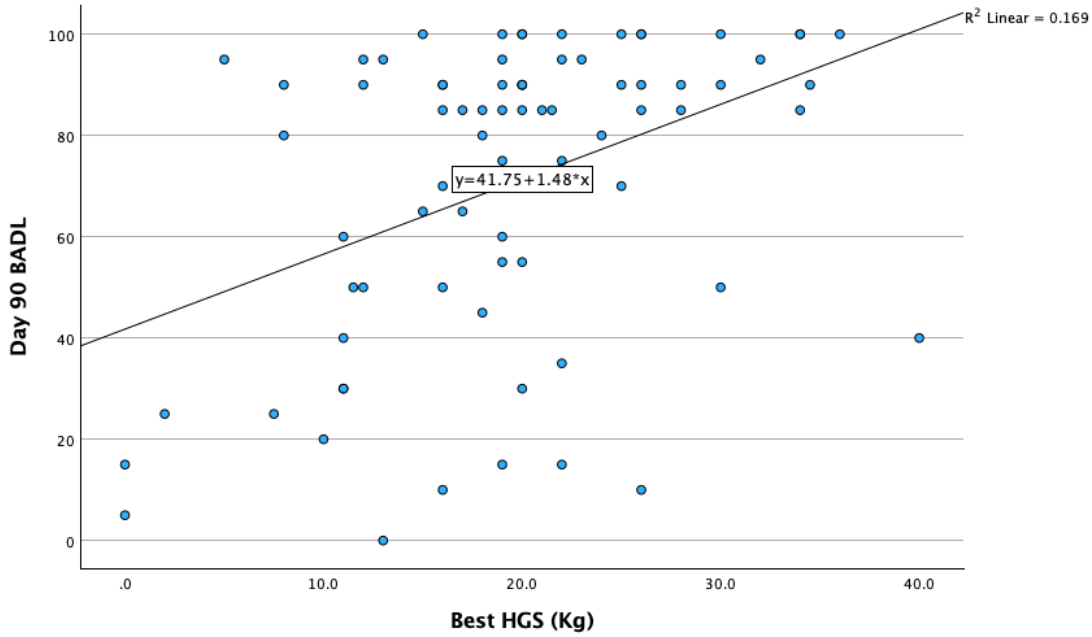


Figure 9.40 Scatter plot *Day 90 Barthels activity of daily living and best hand grip strength (Kg)*

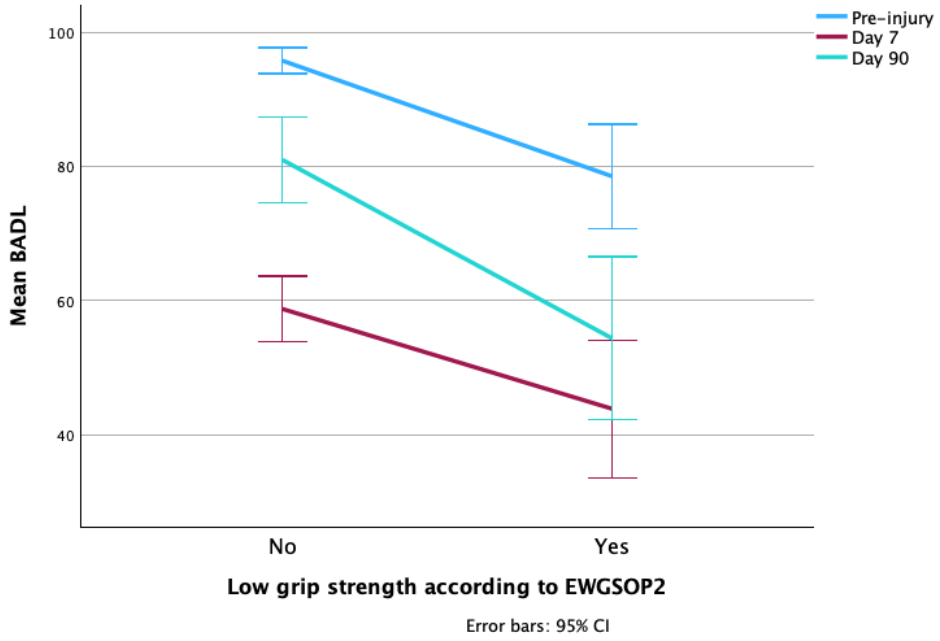


Figure 9.41. *Mean Barthels activity of daily living (pre-injury, day 7 and day 90) with low grip strength according to EWGSOP2 cut-offs*

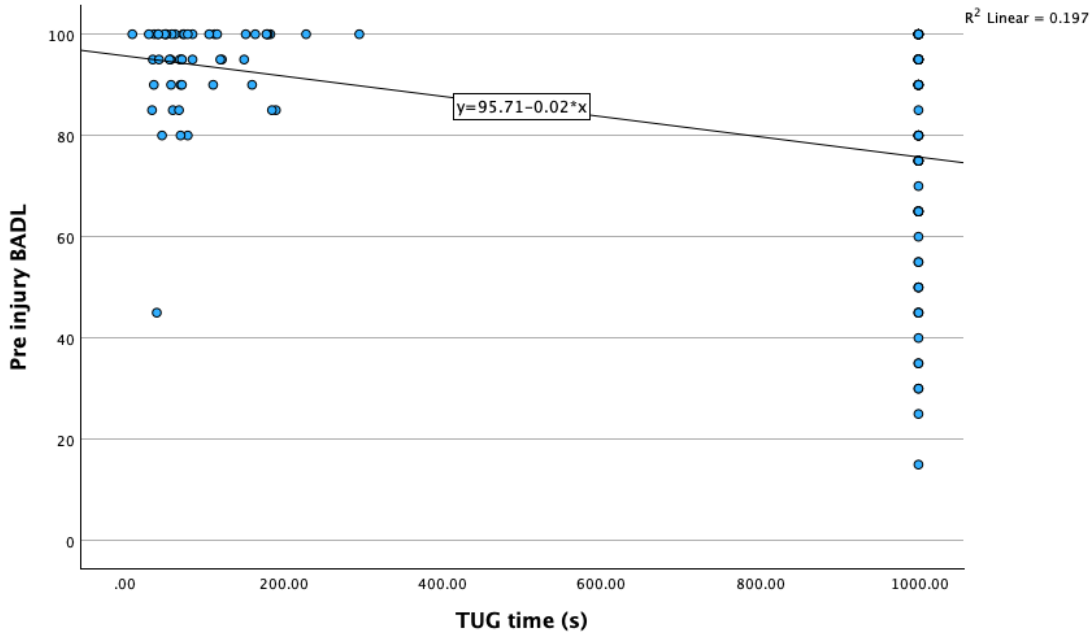


Figure 9.42 Scatter plot *Pre-injury Barthels activity of daily living and Timed up and go test time*

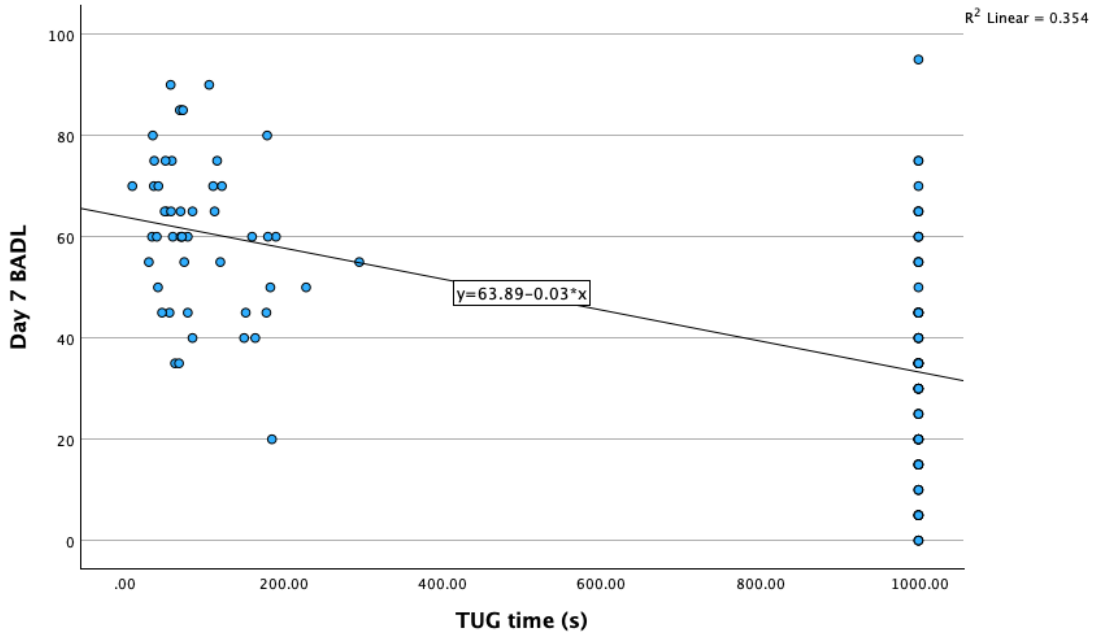


Figure 9.43. *Scatter plot Day 7 Barthels activity of daily living and Timed up and go test time*

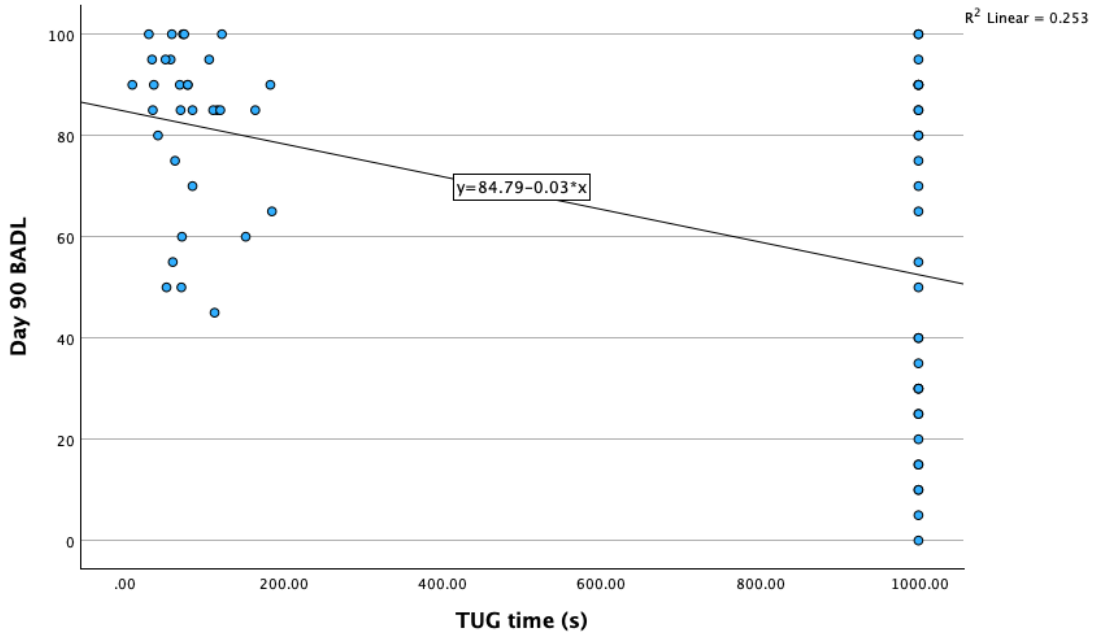


Figure 9.44. *Scatter plot Day 90 Barthels activity of daily living and Timed up and go test time*

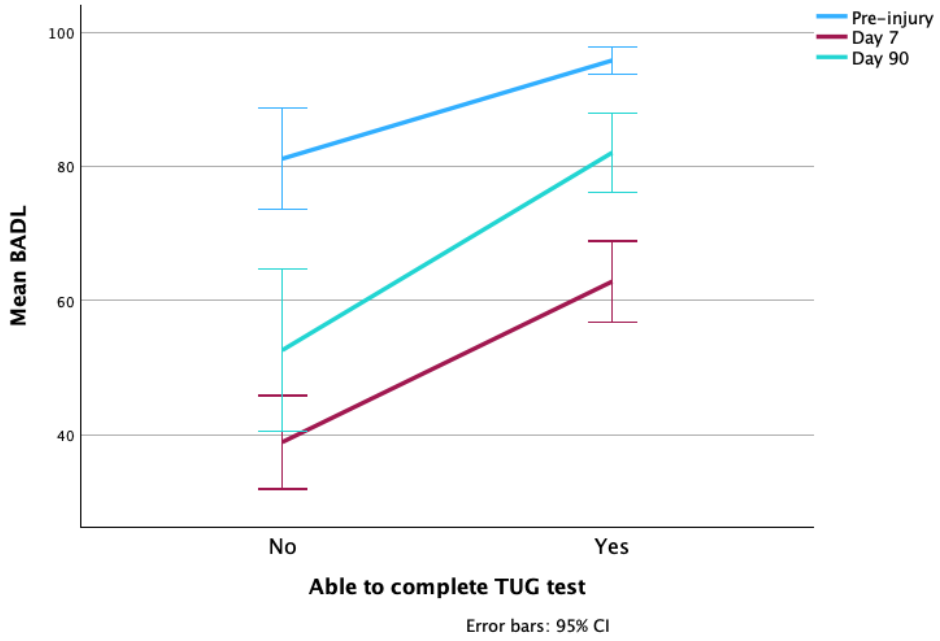


Figure 9.45 *Mean Barthels activity of daily living (pre-injury, day 7 and day 90) and ability to complete Timed up and go test.*

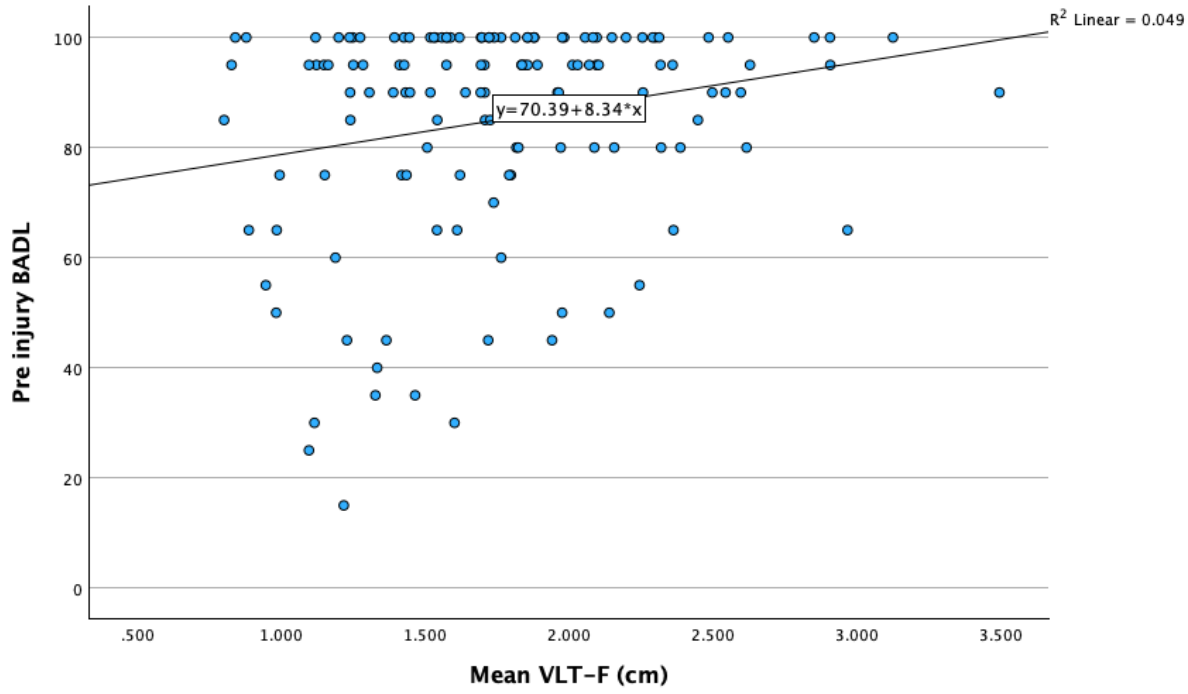


Figure 9.46. *Scatter plot Mean pre-injury BADL and Mean VLT-F*

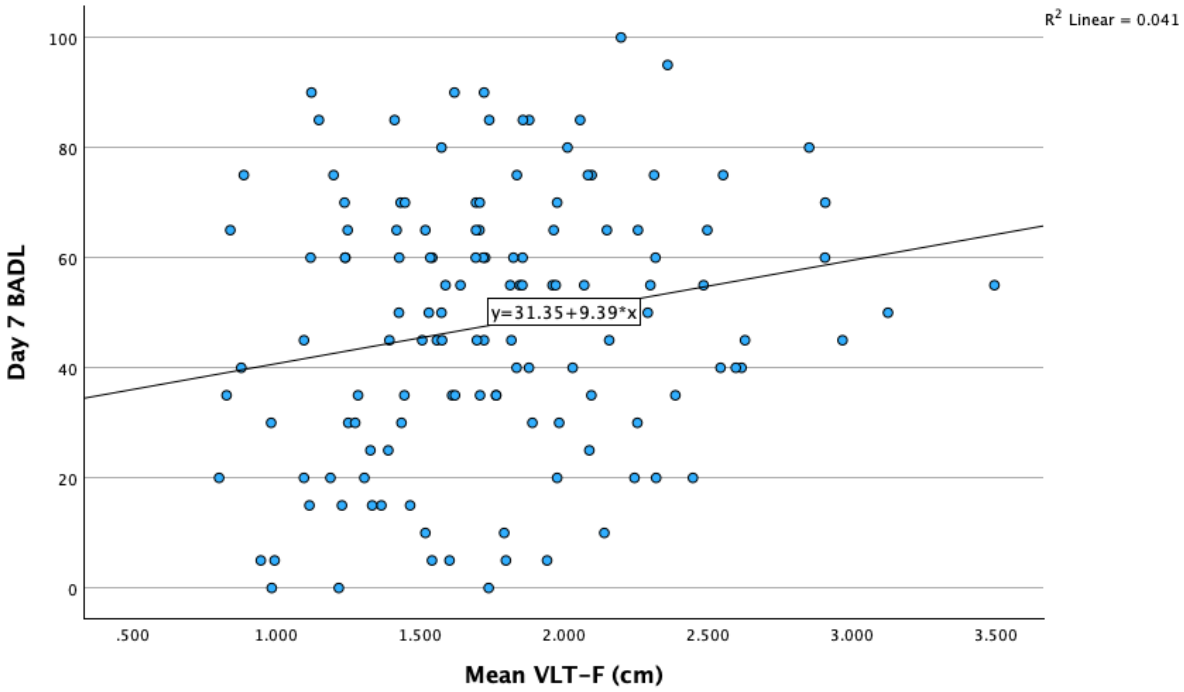


Figure 9.47. *Scatter plot Mean day 7 Barthels activity of daily living and Mean Vastus lateralis thickness fractured leg*

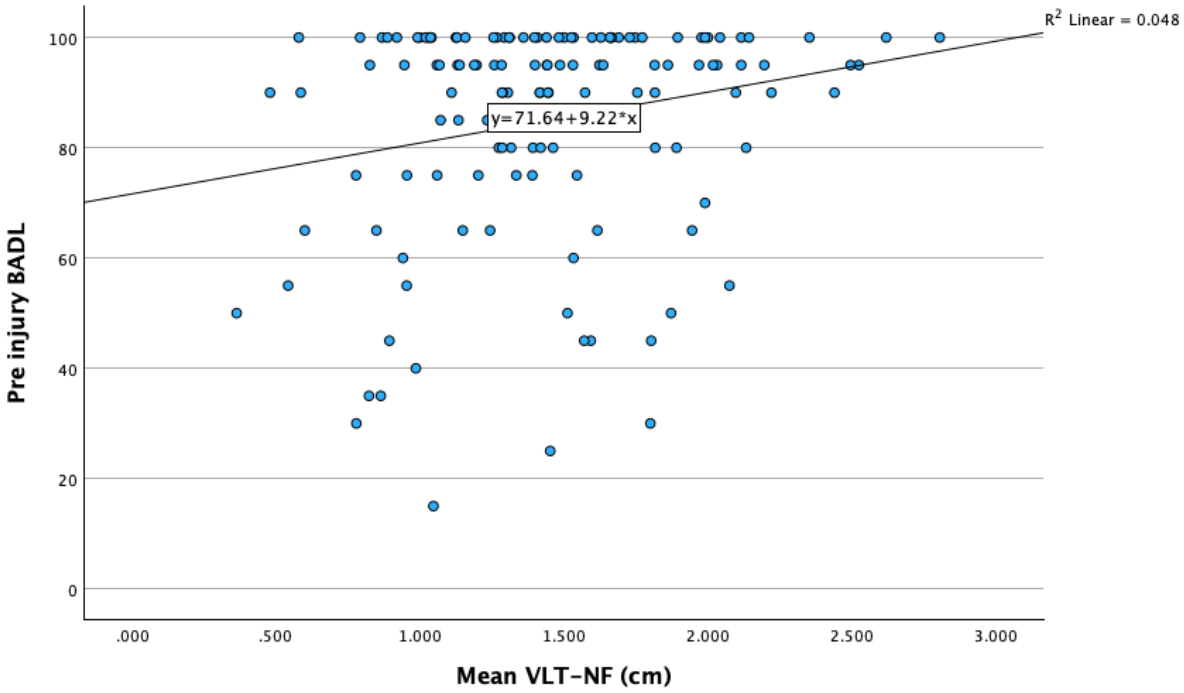


Figure 9.48. Scatter plot *Mean pre-injury Barthels activity of daily living and Mean Vastus lateralis thickness non-fractured leg*

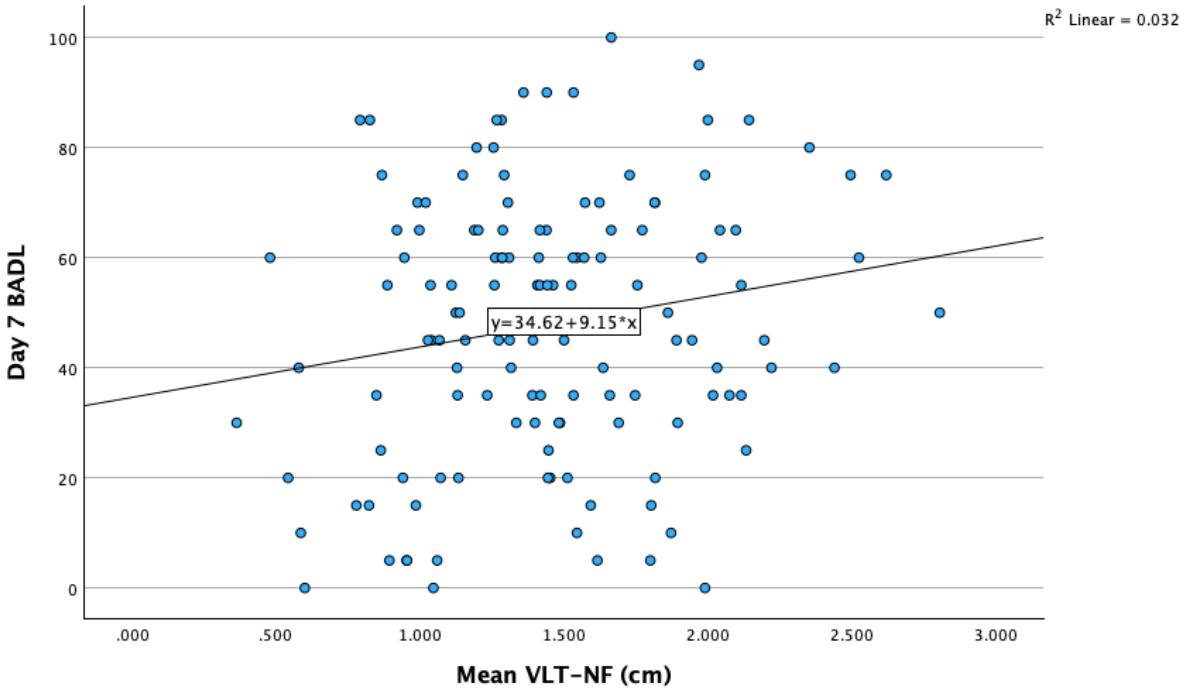


Figure 9.49. *Mean day 7 BADL and Mean VLT-NF*

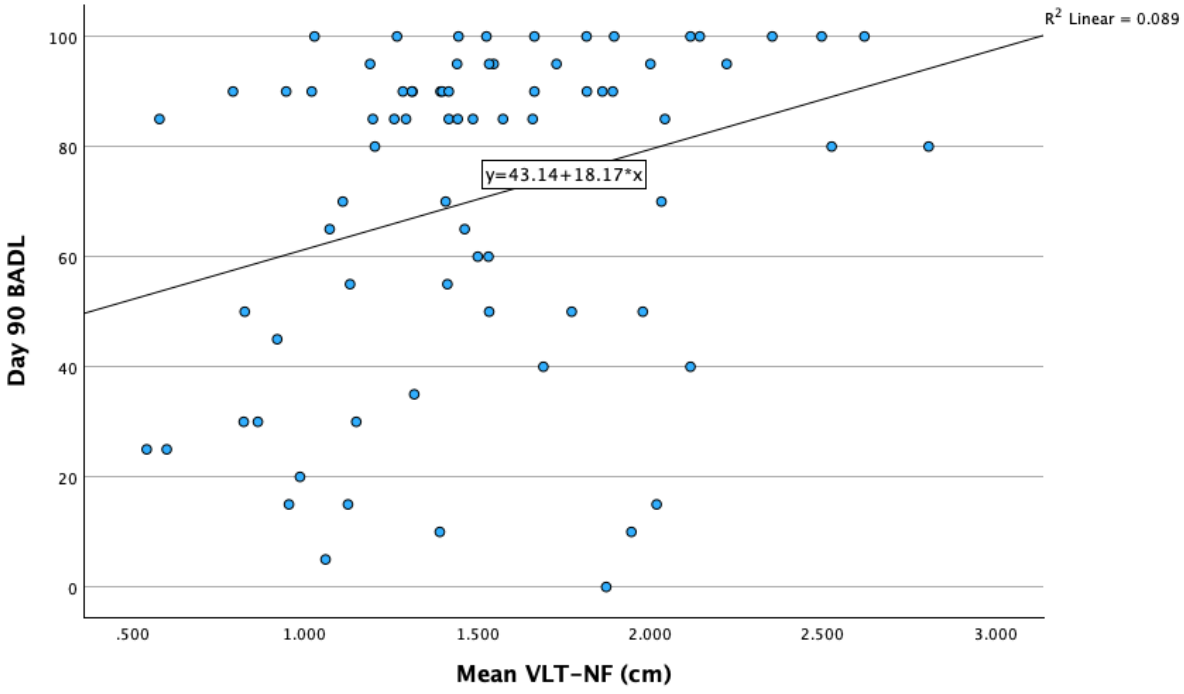


Figure 9.50. *Mean day 90 BADL and Mean VLT-NF*

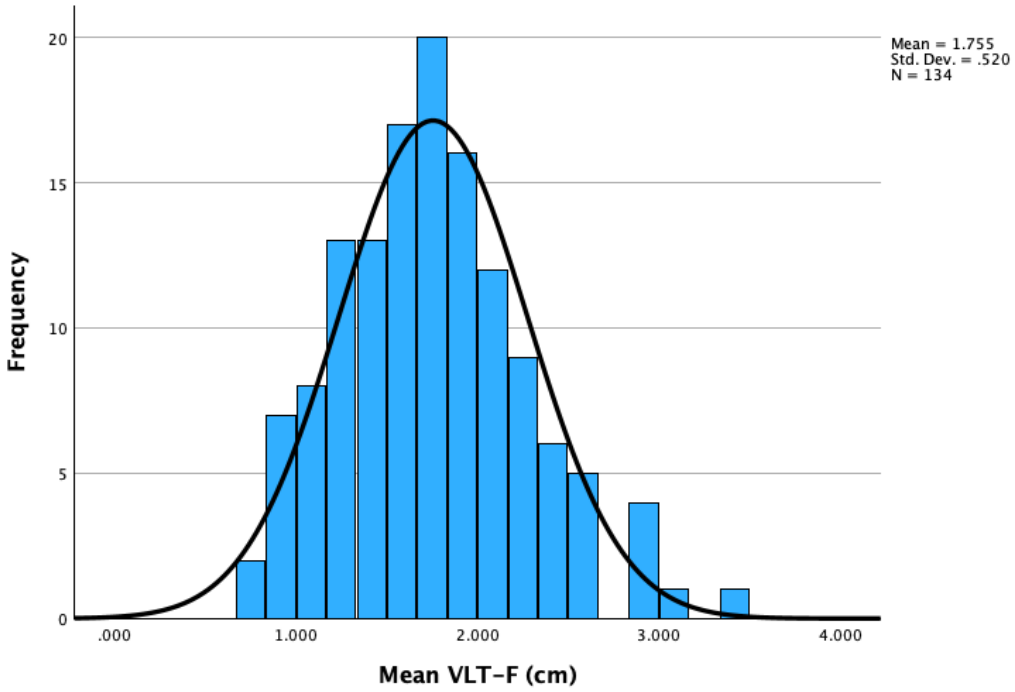


Figure 9.51. *Histogram of frequency of mean VLT-F (cm)*

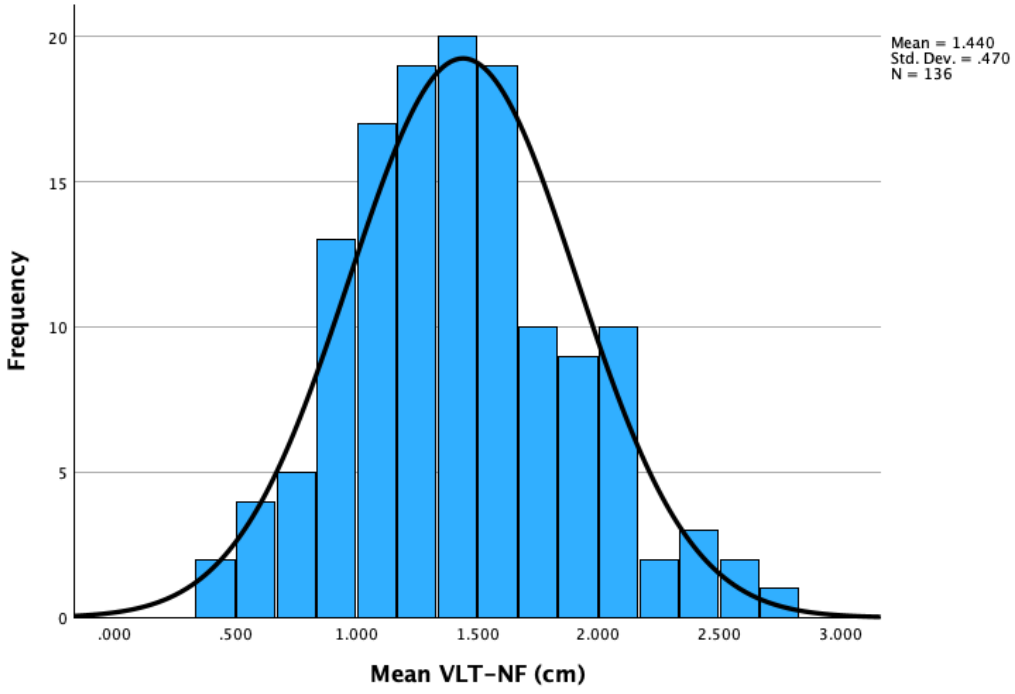


Figure 9.52. *Histogram of frequency of mean VLT-NF (cm)*

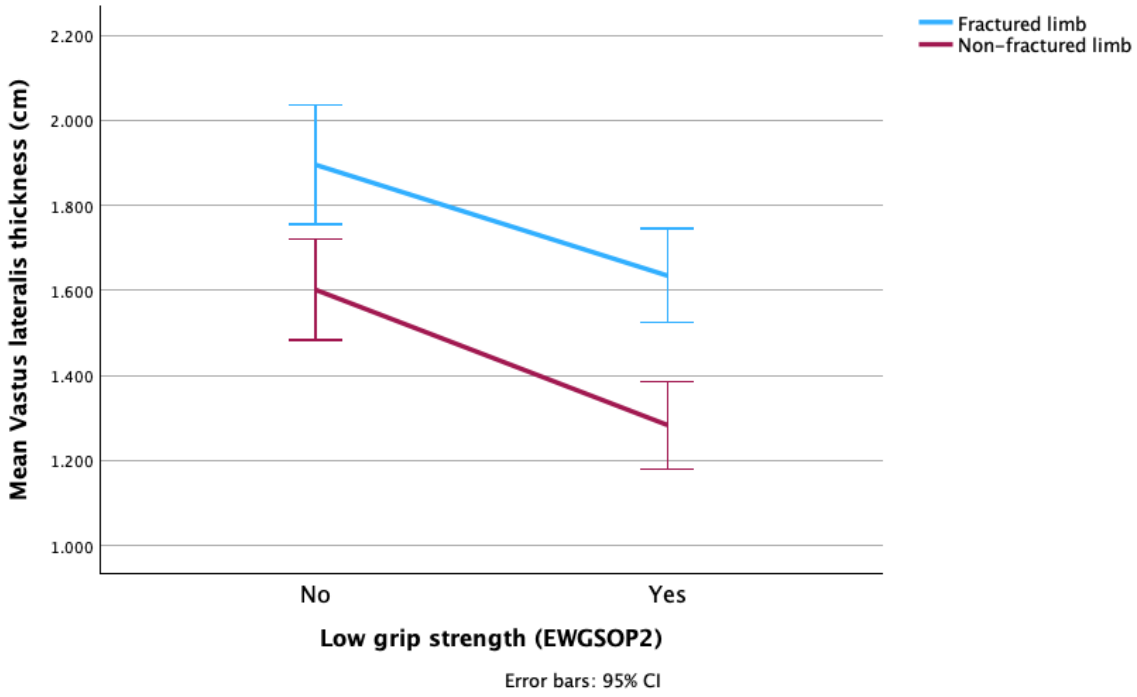


Figure 9.53. *Mean vastus lateralis thickness in both the fractured limb and non-fractured limb and grip strength.*

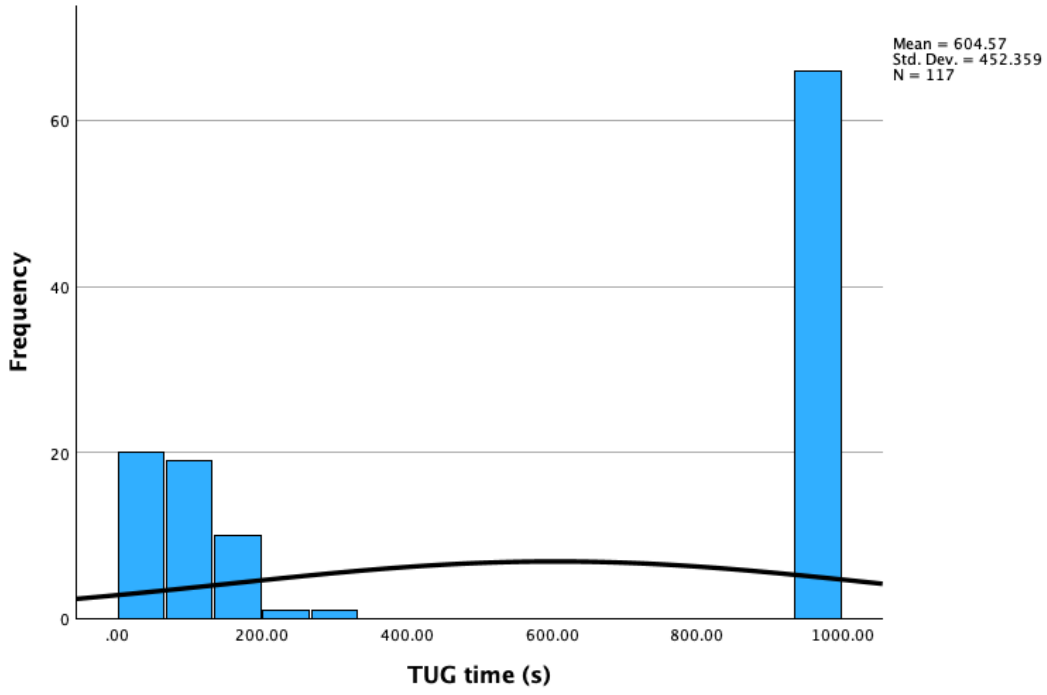


Figure 9.54. *Histogram of frequency and TUG time (s)*

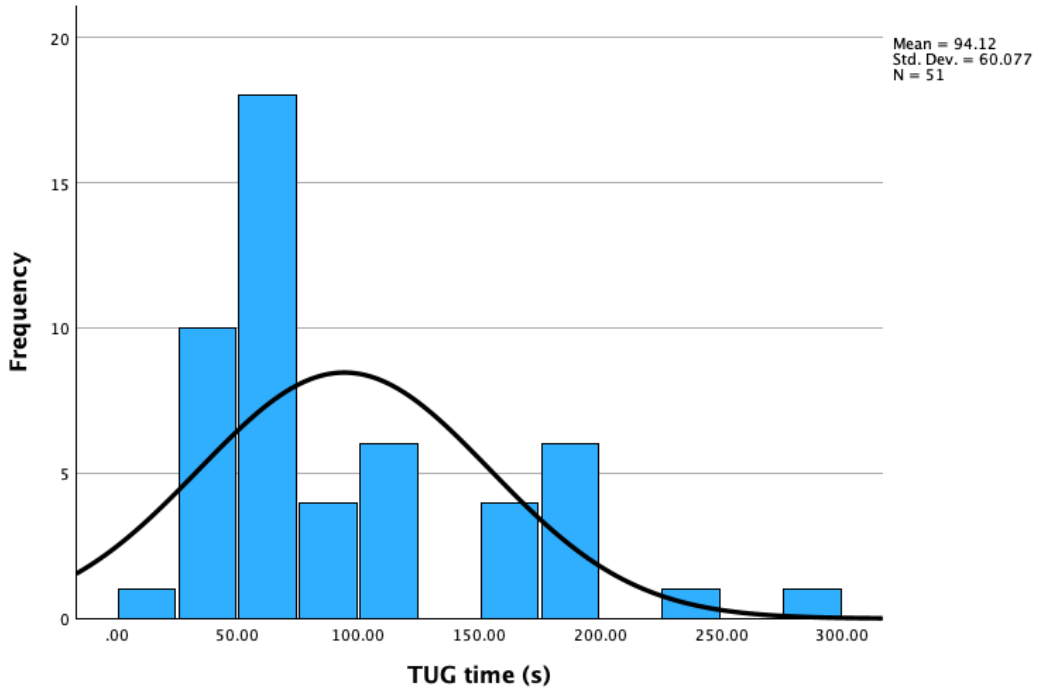


Figure 9.55. *Histogram of frequency and TUG time (s) excluding patients unable to complete the TUG test.*

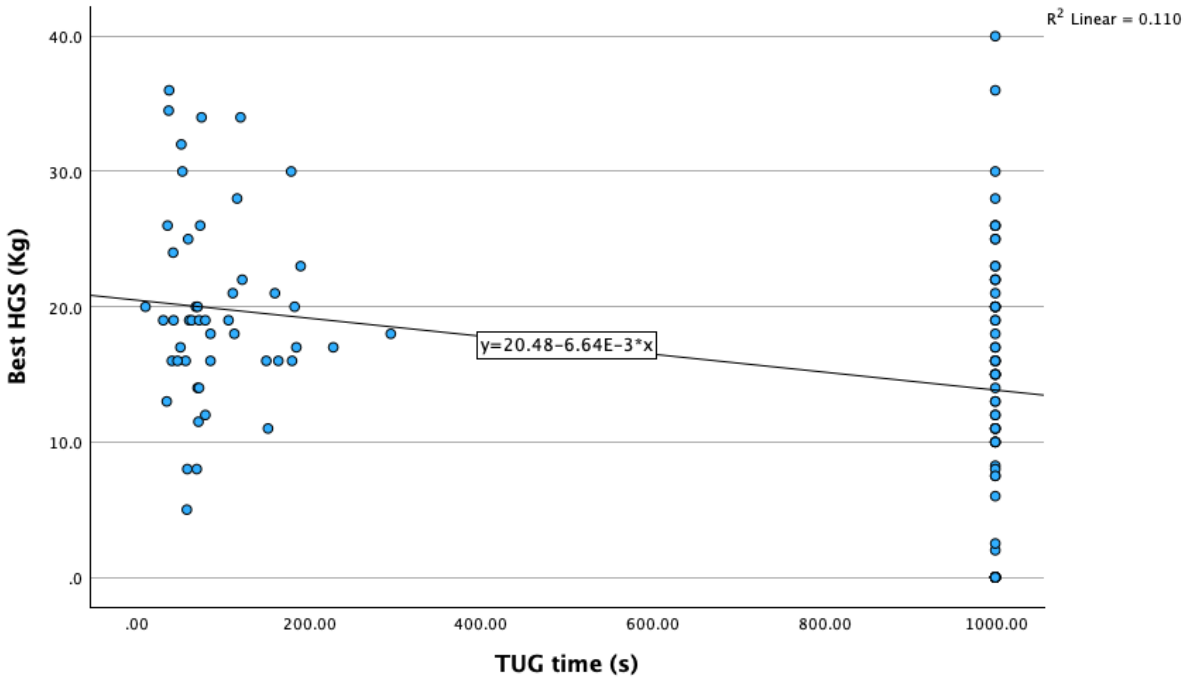


Figure. 9.56. TUG time and best hand grip strength

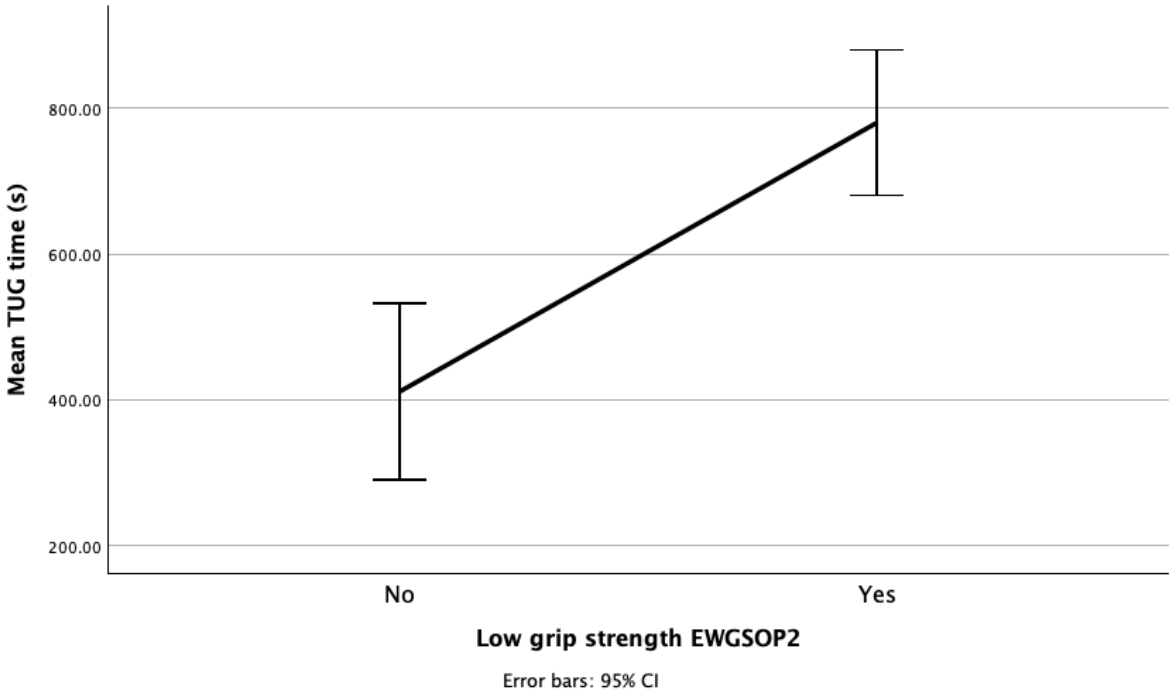


Figure. 9.57. TUG time and low grip strength

HGS

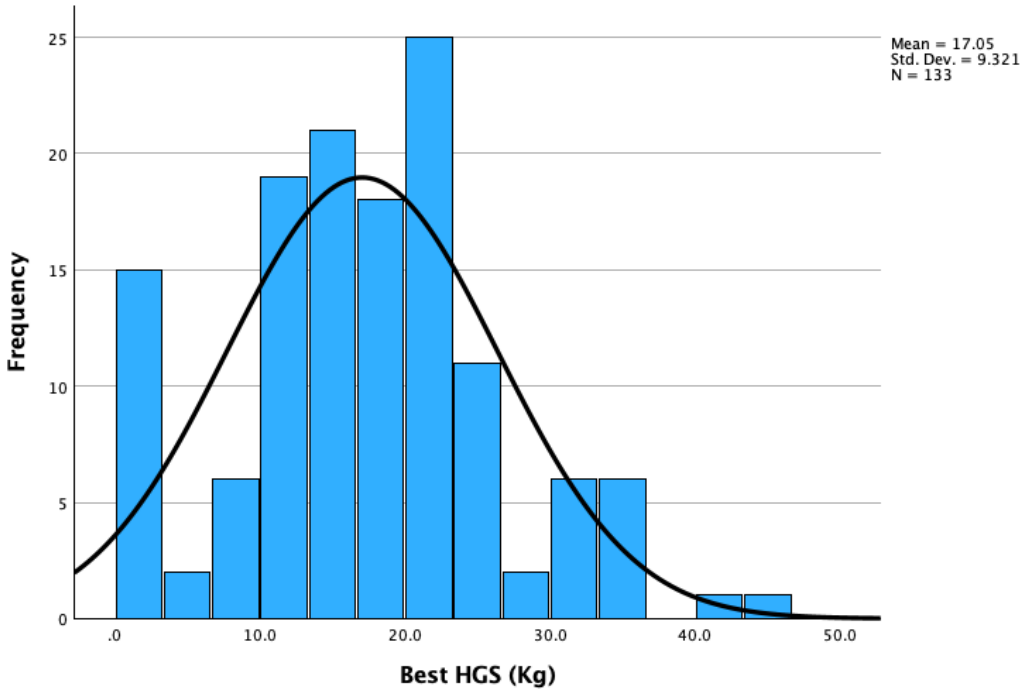


Figure 9.58. *Histogram of frequency and best hand grip strength (Kg)*

9.1.2.10 Regression & ROC tables and survival tables

Logistic regression table relating to table 5.16

Classification Table[a]					
			Predicted		
			Discharged home		Percentage
	Observed		No	Yes	Correct
Step 1	Discharged home	No	29	11	72.5
		Yes	9	45	83.3
	Overall Percentage				78.7
a. The cut value is .500					

Hosmer and Lemeshow Test

Step	Chi-square	df	Sig.
1	3.731	7	.810

Model Summary

Step	-2 Log likelihood	Cox & Snell R Square	Nagelkerke R Square
1	90.325[a]	.332	.446

a. Estimation terminated at iteration number 5 because parameter estimates changed by less than .001.

ROC table and Youden's J Statistic. In relation to table 5.17

Coordinates of the ROC Curve				
Test Result Variable(s)	Positive if Greater Than or Equal To	Sensitivity	1 - Specificity	Youden's Index
D7 BADL	-1.00	1.000	1.000	.000
	2.50	.981	1.000	-.019
	7.50	.963	.947	.016
	15.00	.963	.921	.042
	22.50	.963	.816	.147
	27.50	.963	.763	.200
	32.50	.963	.658	.305

	37.50	.907	.526	.381
	42.50	.852	.421	.431
	47.50	.778	.263	.515
	52.50	.722	.237	.485
	57.50	.630	.211	.419
	62.50	.463	.105	.358
	67.50	.352	.026	.326
	72.50	.241	.026	.214
	77.50	.148	.000	.148
	82.50	.111	.000	.111
	87.50	.056	.000	.056
	92.50	.019	.000	.019
	96.00	.000	.000	.000
Able to complete TUG	-1.00	1.000	1.000	.000
	.50	.759	.184	.575
	2.00	.000	.000	.000

Cox regression tables for continuous variables to predict survival in relation to table 5.19

Case Processing Summary			
		N	Percent
Cases available in analysis	Event[a]	44	31.7%
	Censored	64	46.0%
	Total	108	77.7%
Cases dropped	Cases with missing values	31	22.3%
	Cases with negative time	0	0.0%
	Censored cases before the earliest event in a stratum	0	0.0%
	Total	31	22.3%
Total		139	100.0%

a. Dependent Variable: Days survived

Omnibus Tests of Model Coefficients[a]									
	Overall (score)			Change From Previous Step			Change From Previous Block		
-2 Log Likelihood	Chi-square	df	Sig.	Chi-square	df	Sig.	Chi-square	df	Sig.
357.783	36.678	8	<.001	33.885	8	<.001	33.885	8	<.001

Covariate Means	
	Mean
D7 EQ5D Index	.225
D7 EQ5D Health	50.120
Pre injury RMI	9.889
D7 RMI	2.907
Pre injury BADL	85.278
D7 BADL	46.713
Best HGS	17.072
TUG time	595.688

9.2 Appendices B - Gene expression and outcomes following hip fracture.

9.2.1 FEMUR study gene expression standard operating procedures

9.2.1.1 RNA extraction method for muscle tissue using TRIzol/TRIreagent (Invitrogen/Sigma) SOP038b

Process title: SOP038b: RNA extraction method for MUSCLE TISSUE using TRIzol/TRIreagent (Invitrogen/Sigma) for Greenhaff studies

Location(s) of work: D52/D62	
Substances involved	Hazard
Trizol/TRIreagent (Invitrogen/Sigma)	Toxic if swallowed. Toxic in contact with skin. Toxic if inhaled. Causes severe skin burns & eye damage. May cause respiratory irritation. Suspected of causing genetic defects. May cause damage to organs through prolonged or repeated exposure. Harmful to aquatic life.
Glycogen (20mg/ml)	None
Chloroform	Carcinogen
Isopropanol (Propan-2-ol)	Highly flammable liquid & vapour. Causes serious eye irritation. May cause drowsiness or dizziness.

75% (w/v) Ethanol	Highly flammable liquid and vapour. Causes serious eye irritation.
Equipment involved	Hazard
Polytron Electronic Homogeniser	Electric shock Moving parts hazard Sharps hazard Generates aerosols
Microcentrifuge	Electric shock Moving parts hazard Balance hazard (rotor)
Operations involved	Hazard
Preparing Trizol/Glycogen mix	Trizol - see 'Substances involved'
Homogenising tissue	Handling Human Tissue Electric shock Moving parts hazard Sharps hazard Generates aerosols
Addition of Cholorform/shaking	Chloroform - see 'substances involved'
Spinning down samples in microcentrifuge	Electric shock Moving parts hazard Balance hazard (rotor)
Washing samples	75% Ethanol - see 'substances involved'

Key safety precautions (equipment, PPE, etc.): Use PPE at all times (lab coat, gloves, safety glasses) Working with human tissue – see 'SOP017 Handling Human Body Fluids or Tissue'

All homogenisation to be performed in fumehood to minimise exposure to aerosols.

Take care when using Trizol and– see hazards above.

Ensure all electrical equipment PAT testing certificates are up to date.

Ensure microcentrifuge is balanced before spinning samples.

Wipe up any spillages immediately.

Procedure to be followed:

IMPORTANT: TISSUE MUST BE KEPT FROZEN UNTIL IN TRIZOL

1. Prepare in advance enough glycogen & chloroform:isoamyl alcohol for the number of samples extracting + 1 extra sample (i.e. if extracting 5 samples prepare enough for 6 samples):
    a. Glycogen (Sigma 10µg/µl; dilute 20µg/µl stock 1:1 in H_2O) - 5µl per sample
    b. Chloroform:isoamyl alcohol (49:1 ratio) - 156.8µl chloroform and 3.2µl isoamyl alcohol per sample
2. Pour small amount of liquid nitrogen into metal kidney-shaped tray.
3. Tip out muscle tissue into metal tray and find an appropriate sized piece of muscle (approx. 5 - 35mg).
4. Weigh out muscle tissue in a 2ml microcentrifuge tube – keep tube in liquid nitrogen after weighing.
5. Add 800µl Trizol to sample.
6. Homogenise using Polytron/Electronic homogeniser for 30 seconds (time this).
7. Add 5ul glycogen (10µg/µl) and invert tube several times. Incubate homogenate at room temp for 5 mins.
8. Add 160µl choloroform : isoamyl alcohol (49:1) - see step 1.
9. Cap tube and shake vigorously for 30 seconds, followed by vortex for a few seconds.
10. Incubate at room temp for 2 - 3 mins.
11. Spin at 12,000g for 15 mins at 4^0C.
12. Transfer aqueous phase (approx. 500µl) to a clean 1.5ml microcentrifuge tube. Store organic phase at -80^{o}C in case proteins are needed for subsequent analysis.
13. Add 400µl ice cold isopropanol (propan-2-ol) and leave overnight -20^0C to precipitate.

NEXT DAY

1. Spin samples at 12,000g for 15 mins at 4^0C.
2. Remove supernatant and drain samples over tissue paper for 5 mins.
3. Wash pellet by adding 800µl of 75% EtOH and vortexing for a few seconds.
4. Spin at 10,000g for 10mins.
5. Remove supernatant and air dry over tissue paper for a few minutes.
6. Briefly spin in microfuge and pipette off any remaining EtOH.
7. Re-dissolve pellet in 30µl of RNase-free H_2O.
8. Quantify 10-fold dilutions of the RNA samples using the nanodrop.

Emergency/spillage procedure:

Mop up all spillages with absorbent paper towel and wipe down area with copious amounts of water-soaked absorbent towel.

If Trizol containing is spilt on clothing/skin remove clothing immediately and rinse with copious amounts of water – Polyethylene Glycol 300 (PEG 300) can be used on skin to deactivate any residual phenol – seek first aid/medical advice immediately.

Place all contaminated materials in yellow clinical waste container.

Disposal and decontamination procedure:

Dispose of all materials in yellow clinical waste container.

Clean down all areas used with water-soaked absorbent paper towel followed by 10% Distel.

Storage requirements:

Glycogen – store at -20^0C

Trizol – store at 4^0C

Chloroform – store in organic solvents cabinet at room temp.

75% Ethanol – store in organic solvents cabinet at room temp.

Isopropanol - store in organic solvents cabinet at room temp.

Transport requirements:

Record keeping:

Cross references (COSHH, risk assessments, manuals):

9.2.1.2 First strand cDNA synthesis SOP013b

Process title:
SOP013b: First Strand cDNA Synthesis – Greenhaff studies

Location(s) of work: D52/D62

Substances involved	Hazard
dNTP's	None
Random primers	None
Invitrogen SuperScript III Reverse Transcriptase (200U/µl) - 10,000 Unit kit (Cat no. 18080-044)	None
RNaseOUT™ RNase Inhibitor (40U/µl)	None
Equipment involved	**Hazard**
PCR machine	Burn hazard Electric shock
Operations involved	**Hazard**
Setting up reactions	None
Running samples on PCR machine	Burn hazards Electric shock

Key safety precautions (equipment, PPE, etc.):
Use PPE at all times (lab coat, gloves, safety glasses)
Wipe up any spillages immediately.
Ensure PCR machine PAT test certificate is up to date.

Procedure to be followed:

Use Invitrogen SuperScript III Reverse Transcriptase (200U/µl)

- 10,000 Unit kit (Cat no. 18080-044)

1. Add together in a nuclease-free tube:
    a. total RNA 1.0 ug (calculate ul needed to give 1ug)
    b. Random Primers (0.5µg/µl) 1.0 µl
    c. dNTPs (10mM) 1.0 µl
    d. distilled H_20 up to 13 µl
2. Heat to 65^0C for 5 mins.
3. Incubate on ice for at least 1 min.
4. Briefly spin to collect the contents to the bottom of the tube then add:

5 x First-Strand Buffer 4 µl

0.1M DTT 1 µl

RNaseOUT™ RNase Inhibitor (40U/µl) 1 µl

Superscript III RT (200U/µl) 1 µl

5. Mix by pipetting gently up and down.
6. Incubate at 25^0C for 5 mins.
7. Incubate at 50^0C for 60 mins.

} can program these 3 steps as a cycle on the PCR machine

8. Inactivate by heating at 70^0C for 15 mins.
9. cDNA can now be used as template for PCR.*

Extra solutions required:

RNaseOUT™ Recombinant RNase Inhibitor (Cat no. 10777-019)

100mM dNTPs:

Sigma (DNTP10) - £31.36

or Promega (U1240) - £114 (double amount)

or NEB (N0446S) - £102

or Fermentas (R0181) - £78.20

Emergency/spillage procedure:

Soak up with absorbent paper towel

Disposal and decontamination procedure: Clean down area with water soaked paper towel. Dispose of all contaminated materials in yellow clinical waste containers.

Storage requirements: All components stored at -20^{0}C.
Transport requirements:
Record keeping:
Cross references (COSHH, risk assessments, manuals):

9.2.1.3 Microfluidic gene card design and protocol (ABI) SOP027

Process title:
SOP027: Micro-fluidic Gene Card Design and Protocol (ABI)

Location(s) of work: D52/D62

Substances involved	Hazard
2 x Taqman Universal PCR Mastermix (part No. 4304437) cDNA	None None
Equipment involved	**Hazard**
7900HT Fast Real-Time PCR System (Taqman machine) Heraeus MULTIFUGE 3SR centrifuge Micro Fluidic Card Sealer	Electric shock Moving parts Electric shock Moving parts Balance hazard (rotor) Moving parts
Operations involved	**Hazard**
Preparation of samples for loading Centrifugation of cards Sealing cards Inserting and running 7900HT Taqman machine	None Use of Multifuge 3SR centrifuge (see hazards above) Moving parts (Microfluidic Card sealer) Electric shock Moving parts

Key safety precautions (equipment, PPE, etc.):

Use PPE at all times (lab coat, gloves, safety glasses).

Ensure all electrical equipment PAT testing certificates are up to date.

Ensure centrifuge is balanced before spinning samples.

Take care when using card sealer – moving/sliding parts.

Wipe up any spillages immediately.

Procedure to be followed:

Design and set up for Taqman Low Density Custom Array using Microfluidic Cards (ABI) on a 7900HT Fast Real-Time PCR System (ABI Applied Biosystems):

Designing Cards

- Go to Thermofisher website.
- Login: Username: kostas.tsintzas@nottingham.ac.uk
  Password: ellizoe2
  (security question: pet's name: ILOT)
- Search for: Home › Life Sciences › PCR › Real-Time PCR (qPCR) › Real-Time > PCR Assays › Gene Expression Analysis Using TaqMan Assays › TaqMan Gene Expression Array Cards and Plates
- Clickable link to Taqman Gene Expression Array Cards and Plates

Preparation of sample specific mix (at room temp.)

- Thaw cDNA and vortex.
- Add 50µl of Taqman Universal PCR Mastermix (2x) (part No. 4304437).
- Add 10µl (200ng) of cDNA into an RNase-free eppendorf tube per sample.
- Make total volume of reaction mix up to 100µl with RNase-free water (i.e. 40µl)
- Vortex and then centrifuge (at room temp.)

Loading the cards

- Remove card from fridge and allow the packaging to reach room temperature.
- Remove the card from the packaging and place foil side down on the bench.

- Load 100µl of the reaction mix into the fill port on the left arm of each fill reservoir (the larger of the 2 holes; the other hole is for overspill) starting with No.1 reservoir. Do this very carefully and slowly to avoid any bubbles.
- Centrifuge the card using a MULTIFUGE 3 s-r, Heraeus. Remove one centrifuge bucket and place on the bench with label facing you, insert the card into the card holder (in a vertical position), making sure the reservoir projects upwards out of the card holder and the reaction wells face the same direction as the 'This side Out' label.
- Centrifuge the cards twice for 1 minute (2 x 1 min spins) at 1200 rpm as stated in the manual.

Sealing the cards

- Make sure the carriage (ABI Applied Biosystems, Model 4331770, Rev. A5) is in the start position.
- Place the card foil side up and with fill reservoir end closest to the arrow.
- Push the carriage across the card, do not return the carriage with card in place (but return after removing the card).
- Remove the card.
- Use scissors to remove the fill reservoir (i.e. cut along the right edge of the card).

Running the cards (using 7900HT Fast Real-Time PCR System by ABI Applied Biosystems)

- Open new file using SDS 2.3 software.
- Choose Relative quantification ΔΔCt.
- Choose 384 well Taqman Low Density Custom Array.
- Create a template for the study:

  Put CD that came with plates into PC then File > Import

  Browse to CD drive and import the SDS text file

  File > Save As then give template a name and save as a template file (.sdt)from the dropdown menu.
- Enter the samples ID.
- Open holder, place card and close.
- Start run (~2hrs).

P.T.O.

After the end of the run, you may view data by:

- Open a new file
- Choose 'Relative Quantification ΔΔCt study' from drop down menu and click OK.
- When file is open, add the plate in question (using the 'add plate' command from the drop down menu) into the file for subsequent analysis.

Emergency/spillage procedure:

Wipe up all spillages with absorbent paper towel.

Disposal and decontamination procedure:

Place all contaminated material in yellow clinical waste container.

Wipe down area with 10% Distel.

Storage requirements:

cDNA samples – stored at -20^{0}C

Universal mastermix – stored at 4^{0}C

Transport requirements:

Record keeping:

Cross references (COSHH, risk assessments, manuals):

9.2.2 Gene expression data

9.2.2.1 Gene List

	Gene symbol	Gene	Card_annotation	Function
g1	18S	18S-Hs99999901_s1	Hs99999901_s1	Eukaryotic 18S rRNA
g2	ACO1	ACO1-Hs00158095_m1	Hs00158095_m1	aconitase 1
g3	ACTA1	ACTA1-Hs00559403_m1	Hs00559403_m1	actin, alpha 1, skeletal muscle
g4	ADIPOQ	ADIPOQ-Hs00605917_m1	Hs00605917_m1	adiponectin, C1Q and collagen domain containing
g5	AKT1	AKT1-Hs00178289_m1	Hs00178289_m1	v-akt murine thymoma viral oncogene homolog 1
g6	ALDH2	ALDH2-Hs01007998_m1	Hs01007998_m1	aldehyde dehydrogenase 2 family (mitochondrial)
g7	AMPD1	AMPD1-Hs00163633_m1	Hs00163633_m1	adenosine monophosphate deaminase 1
g8	ATF4	ATF4-Hs00909569_g1	Hs00909569_g1	activating transcription factor 4
g9	ATF6	ATF6-Hs00232586_m1	Hs00232586_m1	activating transcription factor 6
g10	ATG12	ATG12-Hs00740818_m1	Hs00740818_m1	autophagy related 12
g11	ATG9A	ATG9A-Hs01036946_m1	Hs01036946_m1	autophagy related 9A
g12	ATP2A1	ATP2A1-Hs01092295_m1	Hs01092295_m1	ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 1
g13	ATP2A2	ATP2A2-Hs00544877_m1	Hs00544877_m1	ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 2
g14	ATP2B2	ATP2B2-Hs01090453_m1	Hs01090453_m1	ATPase plasma membrane Ca2+ transporting 2
g15	ATP2B4	ATP2B4-Hs00608066_m1	Hs00608066_m1	ATPase plasma membrane Ca2+ transporting 4
g16	ATP5B	ATP5B-Hs00969569_m1	Hs00969569_m1	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide

g17	ATP5I	ATP5I-Hs00273015_m1	Hs00273015_m1	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit E
g18	ATP6AP2	ATP6AP2-Hs00997145_m1	Hs00997145_m1	ATPase H+ transporting accessory protein 2
g19	B2M	B2M-Hs00187842_m1	Hs00187842_m1	beta-2-microglobulin
g20	BECN1	BECN1-Hs00186838_m1	Hs00186838_m1	beclin 1, autophagy related
g21	BRD4	BRD4-Hs04188087_m1	Hs04188087_m1	bromodomain containing 4
g22	CALM1	CALM1-Hs00237233_m1	Hs00237233_m1	calmodulin 1
g23	CALR	CALR-Hs00189032_m1	Hs00189032_m1	calreticulin
g24	CAMK2A	CAMK2A-Hs00947041_m1	Hs00947041_m1	calcium/calmodulin dependent protein kinase II alpha
g25	CAMK4	CAMK4-Hs00174318_m1	Hs00174318_m1	calcium/calmodulin dependent protein kinase IV
g26	CANX	CANX-Hs01558409_m1	Hs01558409_m1	calnexin
g27	CAPN1	CAPN1-Hs00559804_m1	Hs00559804_m1	calpain 1, (mu/I) large subunit
g28	CAPN2	CAPN2-Hs00965097_m1	Hs00965097_m1	calpain 2, (m/II) large subunit
g29	CARM1	CARM1-Hs00406354_m1	Hs00406354_m1	coactivator associated arginine methyltransferase 1
g30	CAS3	CASP3-Hs00234387_m1	Hs00234387_m1	caspase 3
g31	CASP8	CASP8-Hs01018151_m1	Hs01018151_m1	caspase 8, apoptosis-related cysteine peptidase
g32	CASQ1	CASQ1-Hs00154281_m1	Hs00154281_m1	calsequestrin 1
g33	casq2	CASQ2-Hs00154286_m1	Hs00154286_m1	calsequestrin 2
g34	CAT	CAT-Hs00156308_m1	Hs00156308_m1	catalase
g35	CCL19	CCL19-Hs00171149_m1	Hs00171149_m1	chemokine (C-C motif) ligand 19

g36	COL1A1	COL1A1-Hs00164004_m1	Hs00164004_m1	collagen, type I, alpha 1
g37	COL2A1	COL2A1-Hs00264051_m1	Hs00264051_m1	collagen, type II, alpha 1
g38	COl4A1	COL4A1-Hs00266237_m1	Hs00266237_m1	collagen type IV alpha 1 chain
g39	COL6A3	COL6A3-Hs00915125_m1	Hs00915125_m1	collagen type VI alpha 3 chain
g40	COX5A	COX5A-Hs00362067_m1	Hs00362067_m1	cytochrome c oxidase subunit 5A
g41	COX5B	COX5B-Hs00426950_g1	Hs00426950_g1	cytochrome c oxidase subunit Vb
g42	COX6B1	COX6B1-Hs00266375_m1	Hs00266375_m1	cytochrome c oxidase subunit 6B1
g43	CPT1A	CPT1A-Hs00912671_m1	Hs00912671_m1	carnitine palmitoyltransferase 1A (liver)
g44	CPT1B;CHKB-CPT1B	CPT1B;CHKB-CPT1B-Hs00189258_m1	Hs00189258_m1	carnitine palmitoyltransferase 1B (muscle),CHKB-CPT1B readthrough (NMD candidate)
g45	CRTC1	CRTC1-Hs00993064_m1	Hs00993064_m1	CREB regulated transcription coactivator 1
g46	CS	CS-Hs00830726_sH	Hs00830726_sH	citrate synthase
g47	CTSL	CTSL-Hs00964650_m1	Hs00964650_m1	cathepsin L
g48	CUL4A	CUL4A-Hs00757716_m1	Hs00757716_m1	cullin 4A
g49	CYCS	CYCS-Hs01588973_m1	Hs01588973_m1	cytochrome c, somatic
g50	DLAT	DLAT-Hs00898876_m1	Hs00898876_m1	dihydrolipoamide S-acetyltransferase
g51	DLD	DLD-Hs00164401_m1	Hs00164401_m1	dihydrolipoamide dehydrogenase
g52	EIF2S1	EIF2S1-Hs00187953_m1	Hs00187953_m1	eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa
g53	EIF4B	EIF4B-Hs04368067_g1	Hs04368067_g1	eukaryotic translation initiation factor 4B

g54	EIF4E	EIF4E-Hs00854166_g1	Hs00854166_g1	eukaryotic translation initiation factor 4E
g55	EIF4EBP1	EIF4EBP1-Hs00607050_m1	Hs00607050_m1	eukaryotic translation initiation factor 4E binding protein 1
g56	eno3		Hs01549976_m1	fibronectin 1
g57	ERK1		Hs00385075_m1	mitogen-activated protein kinase 3
g58	FABP3	FABP3-Hs00997360_m1	Hs00997360_m1	fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)
g59	FBXO32	FBXO32-Hs01041408_m1	Hs01041408_m1	F-box protein 32
g60	FGF21	FGF21-Hs00173927_m1	Hs00173927_m1	fibroblast growth factor 21
g61	FOXO1	FOXO1-Hs01054576_m1	Hs01054576_m1	forkhead box O1
g62	FOXO3B;FOXO3	FOXO3B;FOXO3-Hs00921424_m1	Hs00921424_m1	forkhead box O3B pseudogene,forkhead box O3
g63	FST	FST-Hs00246256_m1	Hs00246256_m1	follistatin
g64	GAPDH	GAPDH-Hs02786624_g1	Hs02786624_g1	glyceraldehyde-3-phosphate dehydrogenase
g65	Glud1	GLUD1-Hs04981801_m1	Hs04981801_m1	glutamate dehydrogenase 1
g66	GPD2	GPD2-Hs01090711_m1	Hs01090711_m1	glycerol-3-phosphate dehydrogenase 2 (mitochondrial)
g67	GSK3B	GSK3B-Hs01047719_m1	Hs01047719_m1	glycogen synthase kinase 3 beta
g68	HADH	HADH-Hs00997450_m1	Hs00997450_m1	hydroxyacyl-CoA dehydrogenase
g69	HIF1A	HIF1A-Hs00153153_m1	Hs00153153_m1	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)

g70	HK2	HK2-Hs00606086_m1	Hs00606086_m1	hexokinase 2
g71	HMBS	HMBS-Hs00609297_m1	Hs00609297_m1	hydroxymethylbilane synthase
g72	HSPA8	HSPA8-Hs00941880_m1	Hs00941880_m1	heat shock 70kDa protein 8
g73	IDH2	IDH2-Hs00953879_m1	Hs00953879_m1	isocitrate dehydrogenase (NADP(+)) 2, mitochondrial
g74	IER2	IER2-Hs01109355_m1	Hs01109355_m1	immediate early response 2
g75	IFI30	IFI30-Hs00173838_m1	Hs00173838_m1	IFI30, lysosomal thiol reductase
g76	IGBP1	IGBP1-Hs00426831_mH	Hs00426831_mH	immunoglobulin (CD79A) binding protein 1
g77	IGF1	IGF1-Hs01547656_m1	Hs01547656_m1	insulin-like growth factor 1 (somatomedin C)
g78	IGFBP1	IGFBP1-Hs00236877_m1	Hs00236877_m1	insulin like growth factor binding protein 1
g79	IKBKB	IKBKB-Hs00233287_m1	Hs00233287_m1	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
g80	IL6	IL6-Hs00985639_m1	Hs00985639_m1	interleukin 6 (interferon, beta 2)
g81	IL6R	IL6R-Hs01075666_m1	Hs01075666_m1	interleukin 6 receptor
g82	INSR	INSR-Hs00961560_m1	Hs00961560_m1	insulin receptor
g83	IRS1	IRS1-Hs00178563_m1	Hs00178563_m1	insulin receptor substrate 1
g84	ITPR1	ITPR1-Hs00181881_m1	Hs00181881_m1	inositol 1,4,5-trisphosphate receptor type 1
g85	ITPR3	ITPR3-Hs00609948_m1	Hs00609948_m1	inositol 1,4,5-trisphosphate receptor type 3
g86	JKAMP	JKAMP-Hs00255559_m1	Hs00255559_m1	JNK1/MAPK8-associated membrane protein
g87	JPH1	JPH1-Hs00976073_m1	Hs00976073_m1	junctophilin 1
g88	KCNT1	KCNT1-Hs01063050_m1	Hs01063050_m1	potassium sodium-activated channel subfamily T member 1

g89	LDHA	LDHA-Hs01378790_g1	Hs01378790_g1	lactate dehydrogenase A
g90	M6PRBP1	MAP1LC3A-Hs00738808_m1	Hs00998416_m1	perilipin 3
g91	MAP1LC3A	MAPK3-Hs00385075_m1	Hs00738808_m1	microtubule-associated protein 1 light chain 3 alpha
g92	MAPK9	MAPK9-Hs00177102_m1	Hs00177102_m1	mitogen-activated protein kinase 9
g93	MCUR1	MCUR1-Hs00254417_m1	Hs00254417_m1	mitochondrial calcium uniporter regulator 1
g94	MDH2	MDH2-Hs00938918_m1	Hs00938918_m1	malate dehydrogenase 2
g95	MNF1		Hs00276889_m1	mitochondrial nucleoid factor 1
g96	MPC2	MPC2-Hs00209889_m1	Hs00209889_m1	mitochondrial pyruvate carrier 2
g97	MPST	MPST-Hs00560401_m1	Hs00560401_m1	mercaptopyruvate sulfurtransferase
g98	MRF4		Hs00231165_m1	myogenic factor 6
g99	MSTN	MSTN-Hs00976237_m1	Hs00976237_m1	myostatin
g100	MT1A	MT1A-Hs00831826_s1	Hs00831826_s1	metallothionein 1A
g101	MTFR1	MTFR1-Hs00206110_m1	Hs00206110_m1	mitochondrial fission regulator 1
g102	MTOR	MTOR-Hs00234522_m1	Hs00234522_m1	mechanistic target of rapamycin (serine/threonine kinase)
g103	MYBPC1	MYBPC1-Hs00159451_m1	Hs00159451_m1	myosin binding protein C, slow type
g104	Myf5	MYF5-Hs00271574_m1	Hs00271574_m1	myogenic factor 5
g105	MYH1	MYH1-Hs00428600_m1	Hs00428600_m1	myosin heavy chain 1
g106	MYH2	MYH2-Hs00430042_m1	Hs00430042_m1	myosin heavy chain 2
g107	MYH3	MYH3-Hs01074230_m1	Hs01074230_m1	myosin, heavy chain 3, skeletal muscle, embryonic

g108	MYH4	MYH4-Hs00757977_m1	Hs00757977_m1	myosin, heavy chain 4, skeletal muscle
g109	MYL1	MYL1-Hs00984899_m1	Hs00984899_m1	myosin, light chain 1, alkali; skeletal, fast
g110	MYL6	MYL6-Hs00819642_m1	Hs00819642_m1	myosin, light chain 6, alkali, smooth muscle and non-muscle
g111	MYLK2	MYLK2-Hs00263888_m1	Hs00263888_m1	myosin light chain kinase 2
g112	MYLPF	MYLPF-Hs00203261_m1	Hs00203261_m1	myosin light chain, phosphorylatable, fast skeletal muscle
g113	MYLPF		Hs00203261_m1	myosin light chain, phosphorylatable, fast skeletal muscle
g114	MYO5C	MYO5C-Hs00218921_m1	Hs00218921_m1	myosin VC
g115	MYOG	MYOG-Hs01072232_m1	Hs01072232_m1	myogenin (myogenic factor 4)
g116	NAT1	NAT1-Hs04993284_m1	Hs04993284_m1	N-acetyltransferase 1
g117	NAT2	NAT2-Hs00605099_m1	Hs00605099_m1	N-acetyltransferase 2
g118	NDUFB3	NDUFB3-Hs00992988_m1	Hs00992988_m1	NADH:ubiquinone oxidoreductase subunit B3
g119	NDUFB5	NDUFB5-Hs00159582_m1	Hs00159582_m1	NADH:ubiquinone oxidoreductase subunit B5
g120	NFE2L2	NFE2L2-Hs00232352_m1	Hs00232352_m1	nuclear factor, erythroid 2-like 2
g121	NFKB1	NFKB1-Hs00765730_m1	Hs00765730_m1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
g122	NRF1	NRF1-Hs01031046_m1	Hs01031046_m1	nuclear respiratory factor 1
g123	OGDH	OGDH-Hs01081865_m1	Hs01081865_m1	oxoglutarate dehydrogenase
g124	OPTN	OPTN-Hs00184221_m1	Hs00184221_m1	optineurin
g125	OSTN	OSTN-Hs00898258_m1	Hs00898258_m1	osteocrin
g126	OTOP1	OTOP1-Hs01028641_m1	Hs01028641_m1	otopetrin 1

g127	PARKIN	PARK2-Hs01038322_m1	Hs01038322_m1	parkin RBR E3 ubiquitin protein ligase
g128	PAX3	PAX3-Hs00240950_m1	Hs00240950_m1	paired box 3
g129	PAX7	PAX7-Hs00242962_m1	Hs00242962_m1	paired box 7
g130	PDHA1	PDHA1-Hs04998681_m1	Hs04998681_m1	pyruvate dehydrogenase (lipoamide) alpha 1
g131	PDIA2	PDIA2-Hs00429010_m1	Hs00429010_m1	protein disulfide isomerase family A member 2
g132	PDK2	PDK2-Hs00176865_m1	Hs00176865_m1	pyruvate dehydrogenase kinase, isozyme 2
g133	PDK4	PDK4-Hs01037712_m1	Hs01037712_m1	pyruvate dehydrogenase kinase, isozyme 4
g134	PFKM	PFKM-Hs00175997_m1	Hs00175997_m1	phosphofructokinase, muscle
g135	PKM	PKM-Hs00761782_s1	Hs00761782_s1	pyruvate kinase, muscle
g136	POLRMT	POLRMT-Hs04187596_g1	Hs04187596_g1	polymerase (RNA) mitochondrial (DNA directed)
g137	PPARA	PPARA-Hs00947536_m1	Hs00947536_m1	peroxisome proliferator-activated receptor alpha
g138	PPARG	PPARG-Hs00234592_m1	Hs00234592_m1	peroxisome proliferator-activated receptor gamma
g139	PPARGC1A	PPARGC1A-Hs01016719_m1	Hs01016719_m1	peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
g140	PPP3CA	PPP3CA-Hs00174223_m1	Hs00174223_m1	protein phosphatase 3 catalytic subunit alpha
g141	PRKAA1	PRKAA1-Hs01562315_m1	Hs01562315_m1	protein kinase, AMP-activated, alpha 1 catalytic subunit
g142	PRKAA2	PRKAA2-Hs00178903_m1	Hs00178903_m1	protein kinase, AMP-activated, alpha 2 catalytic subunit
g143	PRKAB1	PRKAB1-Hs00272166_m1	Hs00272166_m1	protein kinase AMP-activated non-catalytic subunit beta 1
g144	PSMA1	PSMA1-Hs01027360_g1	Hs01027360_g1	proteasome (prosome, macropain) subunit, alpha type, 1
g145	PSMC1	PSMC1-Hs02386942_g1	Hs02386942_g1	proteasome (prosome, macropain) 26S subunit, ATPase, 1

g146	PSMC4	PSMC4-Hs00197826_m1	Hs00197826_m1	proteasome 26S subunit, ATPase 4
g147	PTEN	PTEN-Hs02621230_s1	Hs02621230_s1	phosphatase and tensin homolog
g148	PYGM	PYGM-Hs00989942_m1	Hs00989942_m1	phosphorylase, glycogen, muscle
g149	RELA	RELA-Hs01042014_m1	Hs01042014_m1	RELA proto-oncogene, NF-kB subunit
g150	RHOA	RHOA-Hs00357608_m1	Hs00357608_m1	ras homolog family member A
g151	RICTOR	RICTOR-Hs00380903_m1	Hs00380903_m1	RPTOR independent companion of MTOR, complex 2
g152	RPS6KB1	RPS6KB1-Hs00356367_m1	Hs00356367_m1	ribosomal protein S6 kinase B1
g153	RPS6KB2	RPS6KB2-Hs00177689_m1	Hs00177689_m1	ribosomal protein S6 kinase, 70kDa, polypeptide 2
g154	RPTOR	RPTOR-Hs00375332_m1	Hs00375332_m1	regulatory associated protein of MTOR, complex 1
g155	RYR1	RYR1-Hs00166991_m1	Hs00166991_m1	ryanodine receptor 1
g156	SDHA	SDHA-Hs00417200_m1	Hs00417200_m1	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)
g157	SIX1	SIX1-Hs00195590_m1	Hs00195590_m1	SIX homeobox 1
g158	SLC39A6	SLC39A6-Hs01086822_m1	Hs01086822_m1	solute carrier family 39 member 6
g159	SMAD	SMAD2-Hs00183425_m1	Hs00183425_m1	SMAD family member 2
g160	SOCS3	SOCS3-Hs01000485_g1	Hs01000485_g1	suppressor of cytokine signaling 3
g161	SOD2	SOD2-Hs00167309_m1	Hs00167309_m1	superoxide dismutase 2, mitochondrial
g162	SREBF1	SREBF1-Hs04984975_m1	Hs04984975_m1	sterol regulatory element binding transcription factor 1
g163	SREBP		Hs04984975_m1	sterol regulatory element binding transcription factor 1
g164	SRF	SRF-Hs01065256_m1	Hs01065256_m1	serum response factor

g165	STAT1	STAT1-Hs01013996_m1	Hs01013996_m1	signal transducer and activator of transcription 1, 91kDa
g166	STAT3	STAT3-Hs01047580_m1	Hs01047580_m1	signal transducer and activator of transcription 3 (acute-phase response factor)
g167	STIM1	STIM1-Hs00963373_m1	Hs00963373_m1	stromal interaction molecule 1
g168	TFAM	TFAM-Hs01073349_g1	Hs01073349_g1	transcription factor A, mitochondrial
g169	TFB2M	TFB2M-Hs00915025_m1	Hs00915025_m1	transcription factor B2, mitochondrial
g170	TGFB1	TGFB1-Hs00998133_m1	Hs00998133_m1	transforming growth factor, beta 1
g171	TJP2	TJP2-Hs00910543_m1	Hs00910543_m1	tight junction protein 2
g172	TLR4	TLR4-Hs00152939_m1	Hs00152939_m1	toll-like receptor 4
g173	TNC	TNC-Hs01115665_m1	Hs01115665_m1	tenascin C
g174	TNFR1	TNFRSF1A-Hs01042313_m1	Hs01042313_m1	TNF receptor superfamily member 1A
g175	TNNC2	TNNC2-Hs01590581_m1	Hs01590581_m1	troponin C2, fast skeletal type
g176	TNNT1	TNNT1-Hs00162848_m1	Hs00162848_m1	troponin T1, slow skeletal type
g177	TRAF3	TRAF3-Hs00936781_m1	Hs00936781_m1	TNF receptor-associated factor 3
g178	TRIM63	TRIM63-Hs00822397_m1	Hs00822397_m1	tripartite motif containing 63, E3 ubiquitin protein ligase
g179	TRPM1	TRPM1-Hs00931865_m1	Hs00931865_m1	transient receptor potential cation channel subfamily M member 1
g180	TRPM7	TRPM7-Hs00559080_m1	Hs00559080_m1	transient receptor potential cation channel subfamily M member 7
g181	TTN	TTN-Hs00399225_m1	Hs00399225_m1	titin
g182	TUFM	TUFM-Hs00607042_gH	Hs00607042_gH	Tu translation elongation factor, mitochondrial
g183	UCP1	UCP1-Hs00222453_m1	Hs00222453_m1	uncoupling protein 1 (mitochondrial, proton carrier)

g184	UCP3	UCP3-Hs01106052_m1	Hs01106052_m1	uncoupling protein 3 (mitochondrial, proton carrier)
g185	YWHAZ		Hs01122442_m1	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation
g186	ZFAND5	ZFAND5-Hs00826311_m1	Hs00826311_m1	zinc finger AN1-type containing 5
g187	NDUFS6	NDUFS6-Hs00190035_m1	Hs00190035_m1	NADH:ubiquinone oxidoreductase subunit S6
g188	SCNN1A	SCNN1A-Hs00168906_m1	Hs00168906_m1	sodium channel epithelial 1 alpha subunit
g189	CTNNAL1	CTNNAL1-Hs00972098_m1	Hs00972098_m1	alpha catenin
g190	PDPK1	PDPK1-Hs00928927_m1	Hs00928927_m1	3-phosphoinositide-dependent protein kinase 1
g191	PIK3R5	PIK3R5-Hs01046353_m1	Hs01046353_m1	phosphoinositide-3-kinase regulatory subunit 5
g192	SLC2A4	SLC2A4-Hs00168966_m1	Hs00168966_m1	solute carrier family 2 member 4/Glut4 transporter

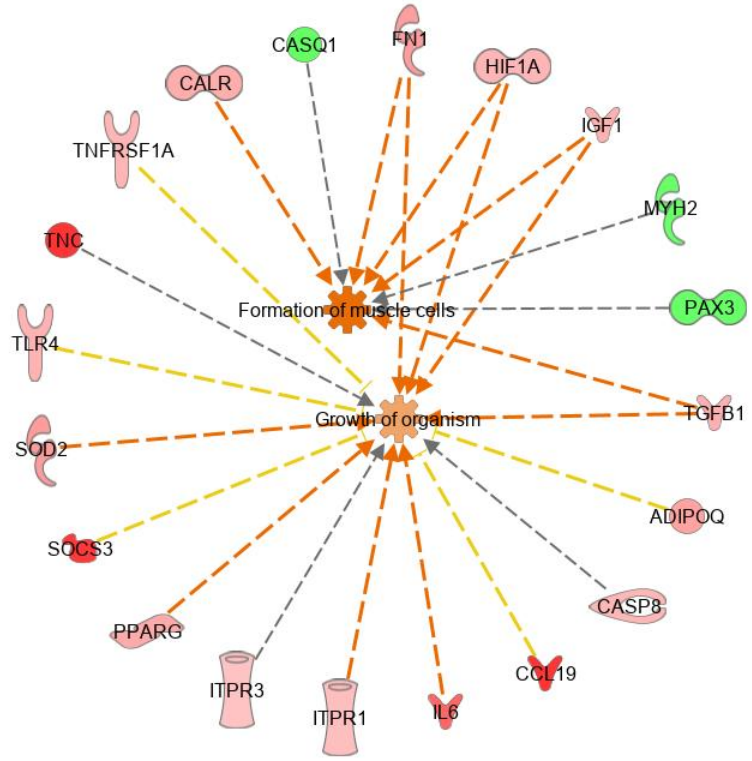
9.2.2.2 Differentially expressed genes when comparing fractured limb (n=76) with non-fractured limb (n=77)

Symbol	Entrez Gene Name	Expr Log Ratio	Expr p-value
ADIPOQ	adiponectin, C1Q and collagen domain containing	0.902	0.013
AMPD1	adenosine monophosphate deaminase 1	-0.627	0.000489
ATP2A1	ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 1	-0.704	0.0024
ATP2B2	ATPase plasma membrane Ca2+ transporting 2	-0.618	0.0069
CALR	calreticulin	0.795	6.37E-09
CAMK2A	calcium/calmodulin dependent protein kinase II alpha	-0.588	0.000259
CASP8	caspase 8	0.693	0.00000108
CASQ1	calsequestrin 1	-0.718	0.00011
CCL19	C-C motif chemokine ligand 19	1.987	0.00000317
COL1A1	collagen type I alpha 1 chain	1.334	7.22E-08
COL6A3	collagen type VI alpha 3 chain	0.933	1.87E-11
CTSL	cathepsin L	0.861	9.41E-08
FN1	fibronectin 1	0.956	0.000000687
HIF1A	hypoxia inducible factor 1 subunit alpha	0.712	0.000000404
HK2	hexokinase 2	1.109	0.000000148
IFI30	IFI30 lysosomal thiol reductase	1.454	7.95E-10
IGF1	insulin like growth factor 1	0.751	0.00000513
IL6	interleukin 6	1.496	0.000000175
ITPR1	inositol 1,4,5-trisphosphate receptor type 1	0.631	0.000000286
ITPR3	inositol 1,4,5-trisphosphate receptor type 3	0.583	0.0033
MT1A	metallothionein 1A	1.948	7.06E-09
MYH2	myosin heavy chain 2	-0.686	0.0038
MYL11	myosin light chain 11	-0.631	0.0018
MYL6	myosin light chain 6	0.635	0.00000222
MYLK2	myosin light chain kinase 2	-1.041	0.00000166
PAX3	paired box 3	-0.698	0.000559
PIK3R5	phosphoinositide-3-kinase regulatory subunit 5	1.076	0.000000041

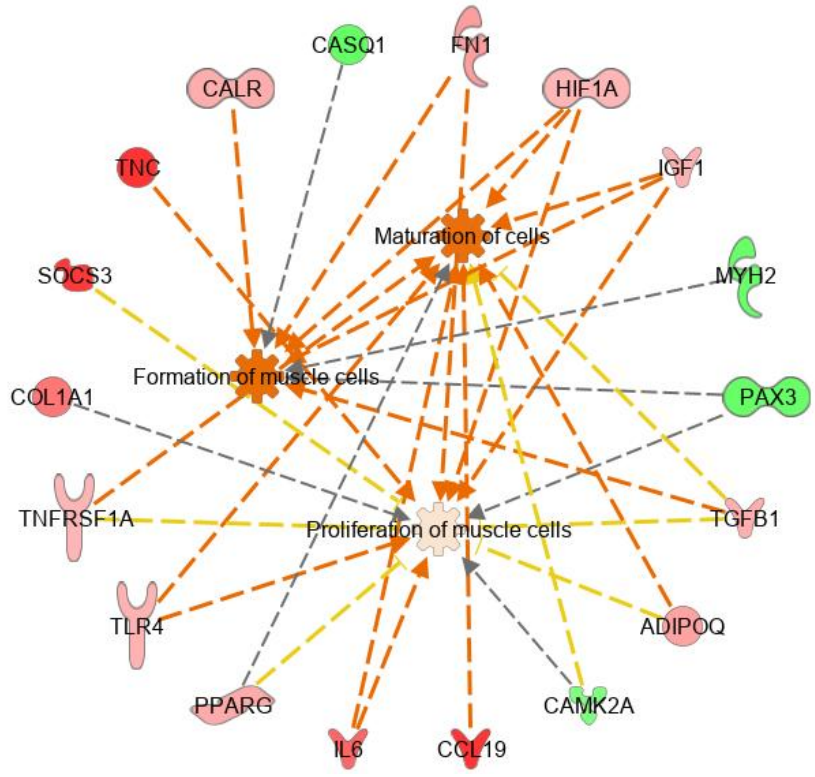
PPARG	peroxisome proliferator activated receptor gamma	0.828	0.000337
SOCS3	suppressor of cytokine signalling 3	1.936	2.74E-13
SOD2	superoxide dismutase 2	0.97	0.0000195
TGFB1	transforming growth factor beta 1	0.783	7.73E-09
TLR4	toll like receptor 4	0.74	1.25E-09
TNC	tenascin C	2.012	0.000000117
TNFRSF1A	TNF receptor superfamily member 1A	0.713	1.21E-09
TNNC2	troponin C2, fast skeletal type	-0.583	0.0026

9.2.2.3 Gene expression rosettes when comparing the fractured limb with the non-fractured limb predictions made by IPA.

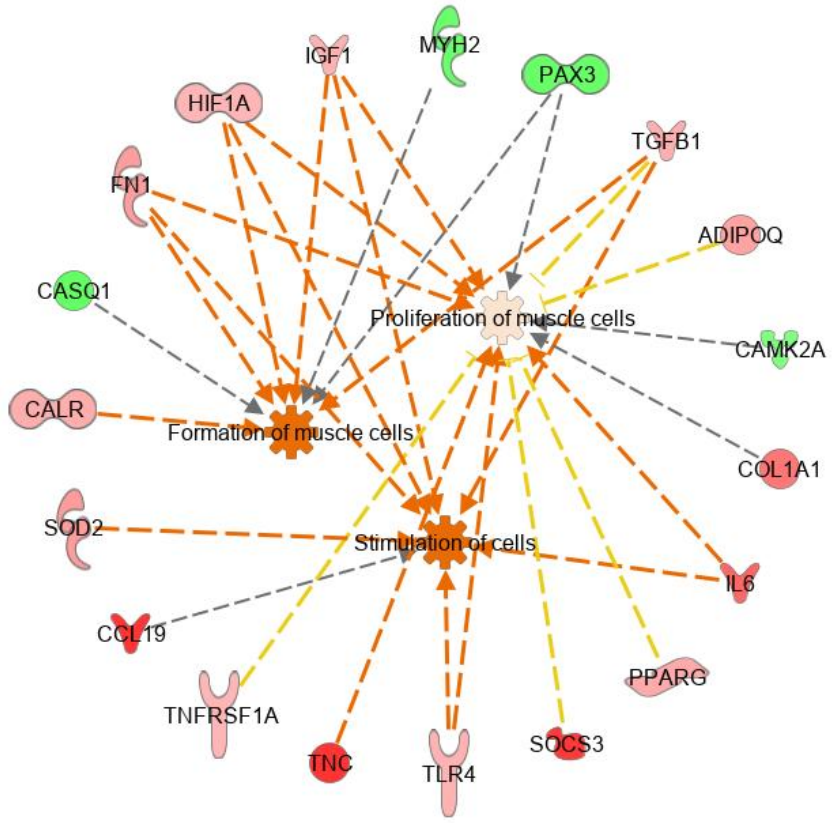
Organismal Development



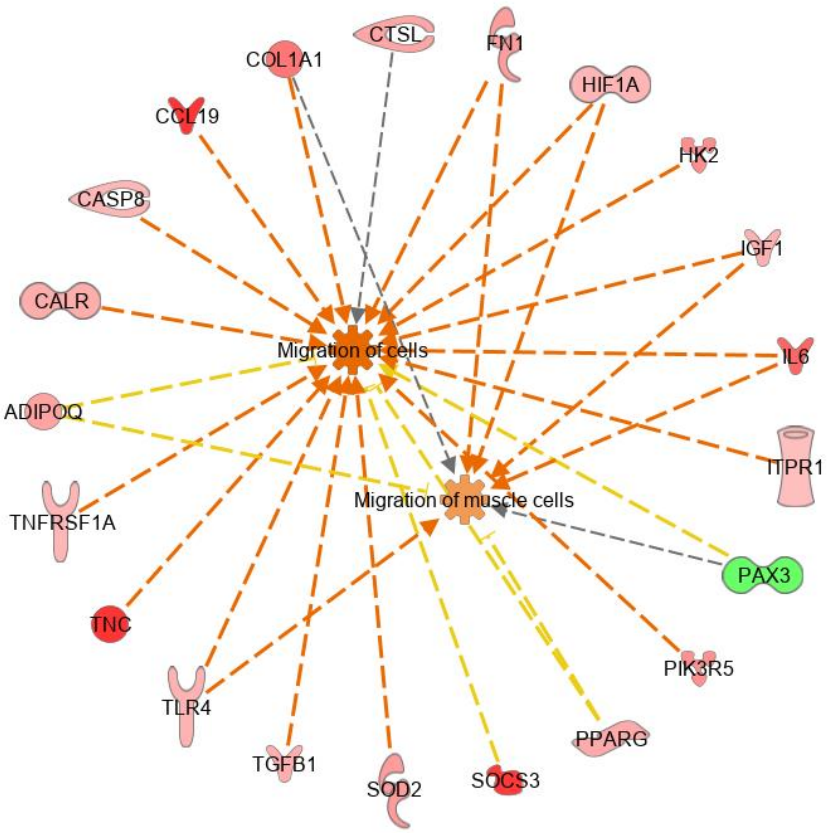
Cellular development



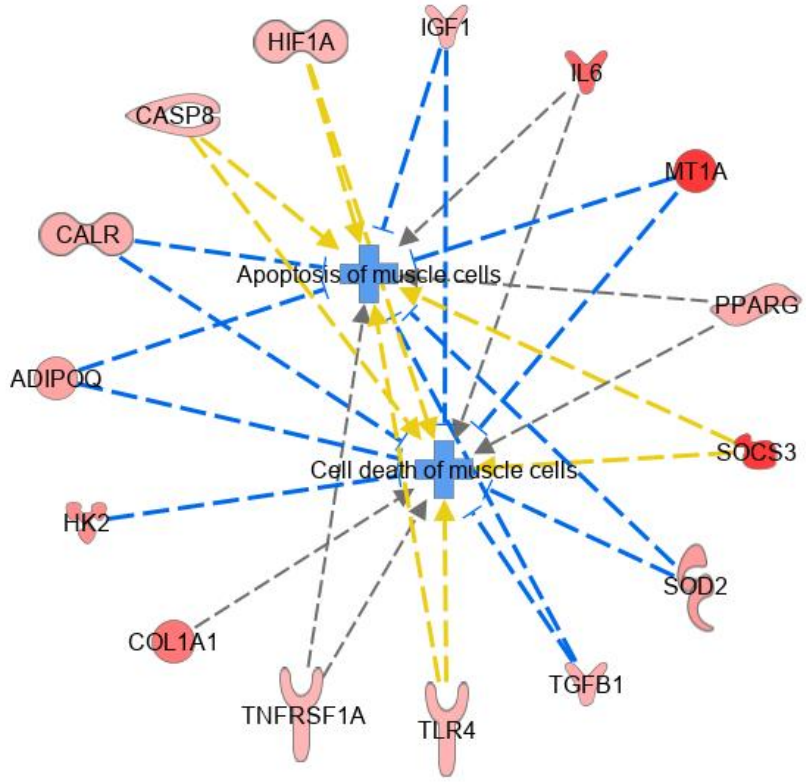
Cellular growth and proliferation



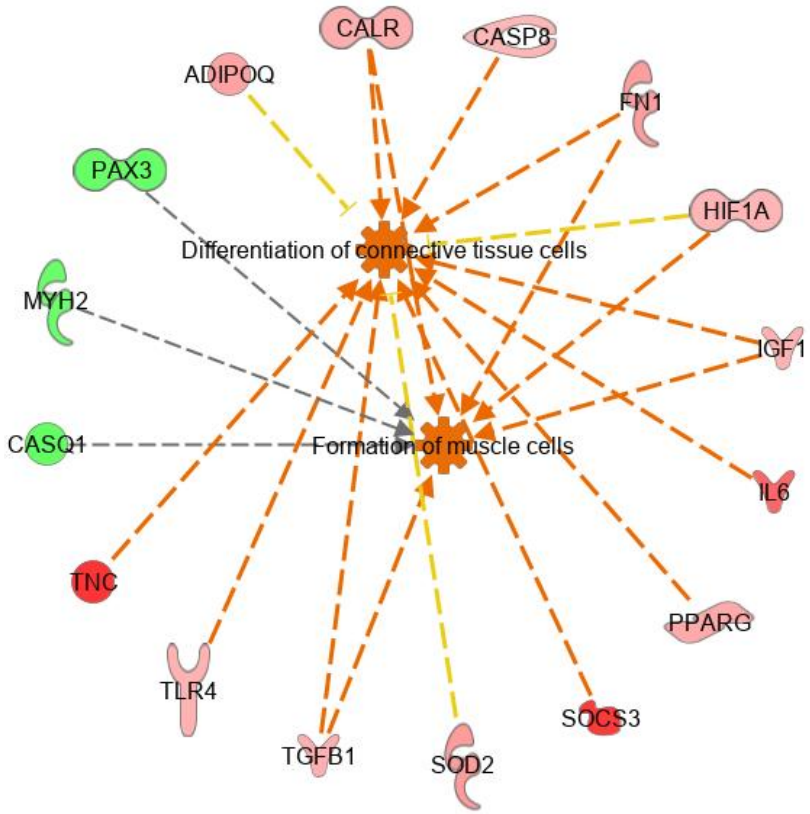
Cellular movement



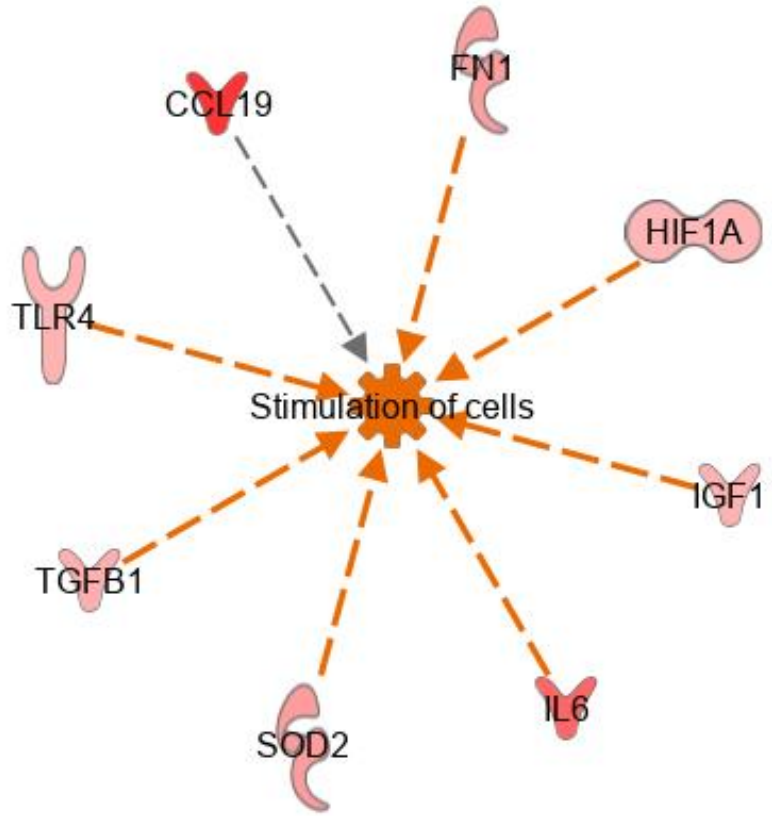
Skeletal and Muscular system development



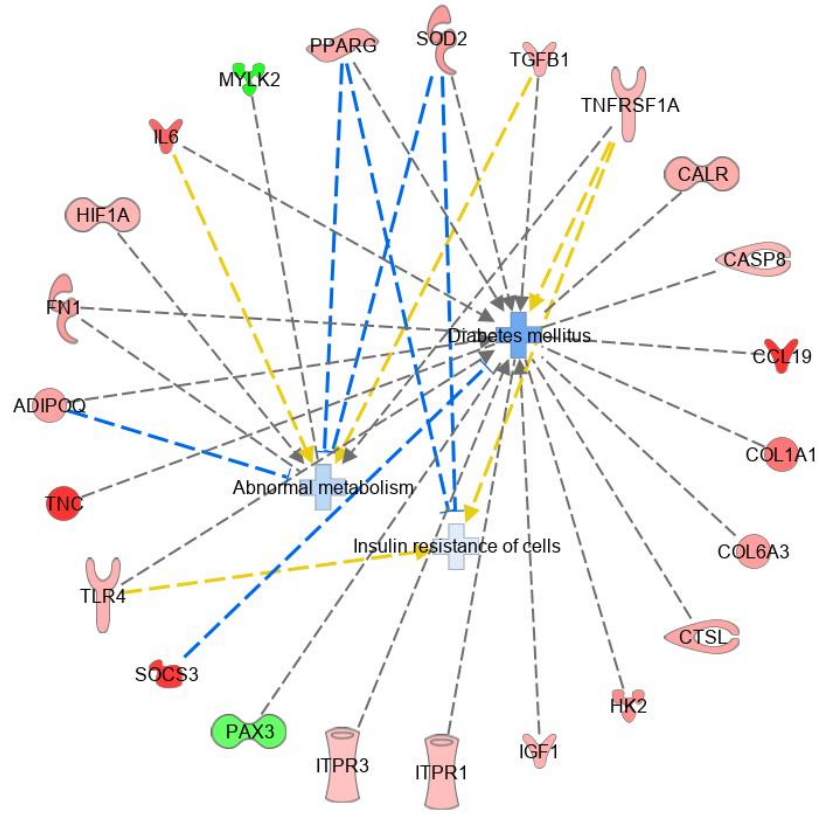
Tissue development



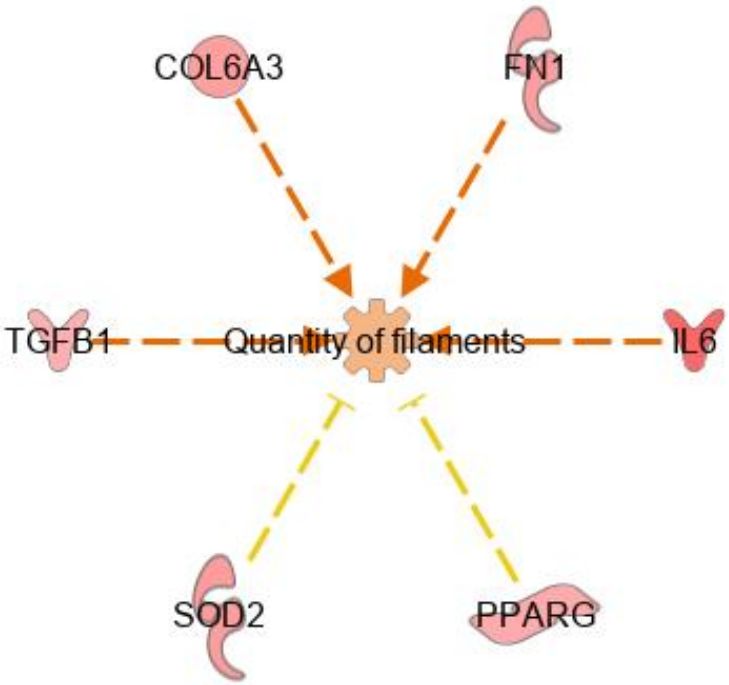
Cell to cell signalling and interaction



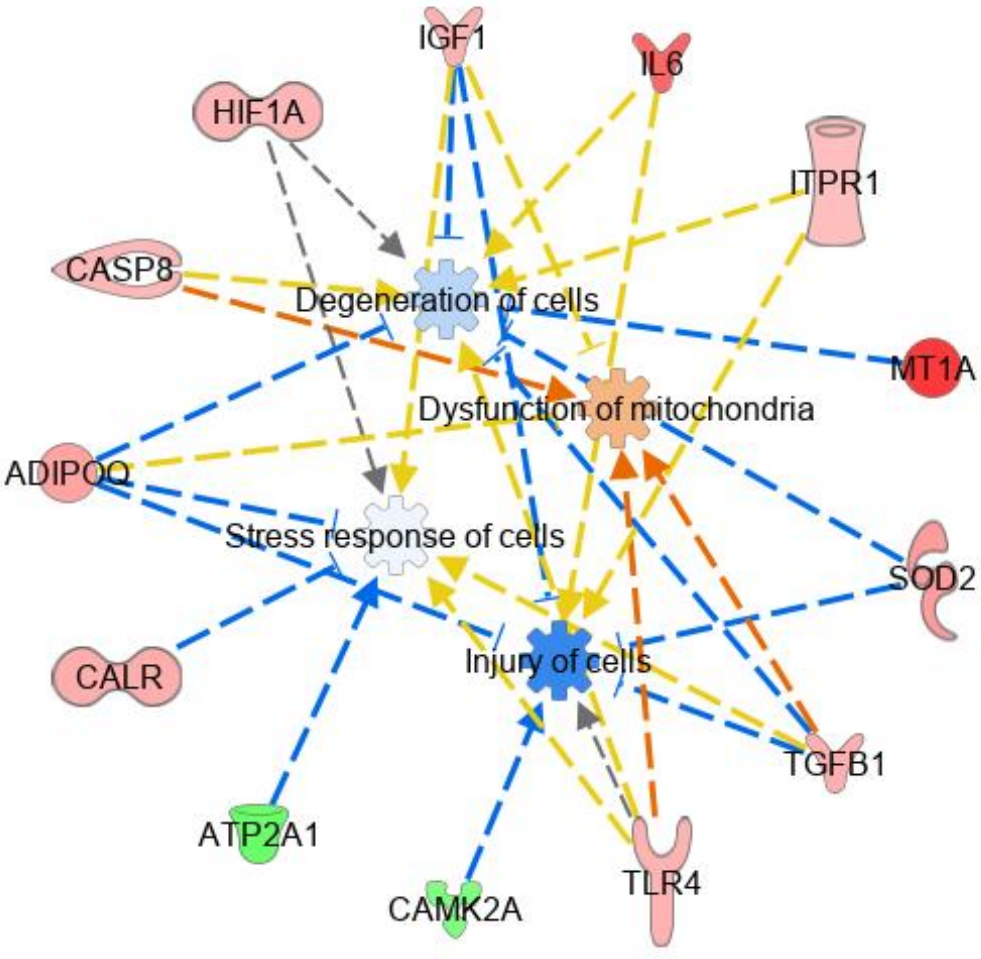
Metabolic disease



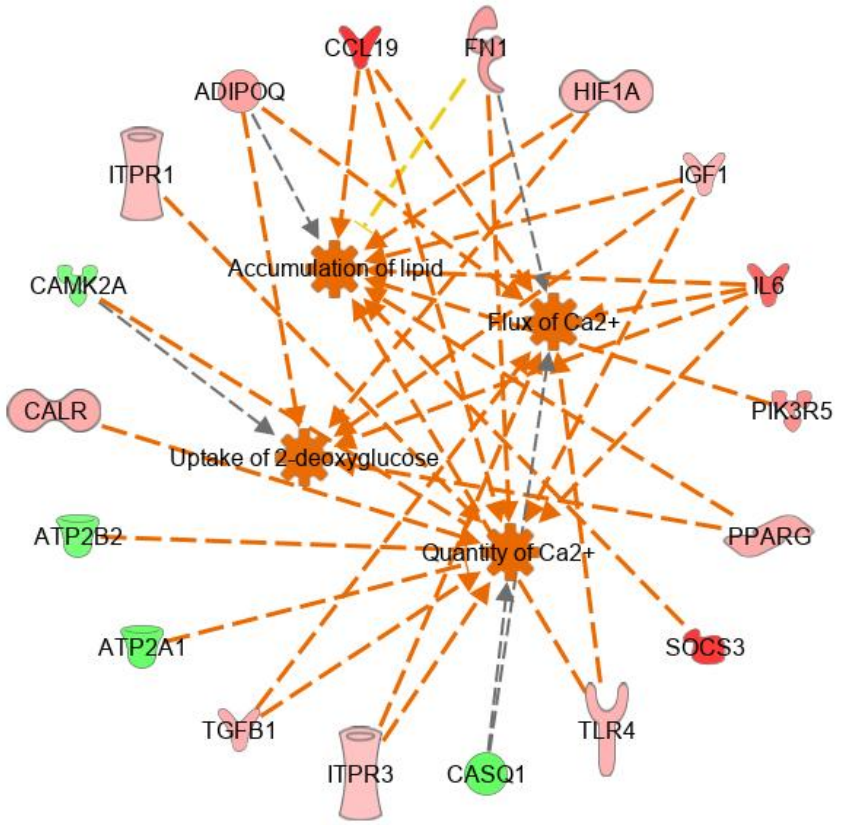
Cellular assembly



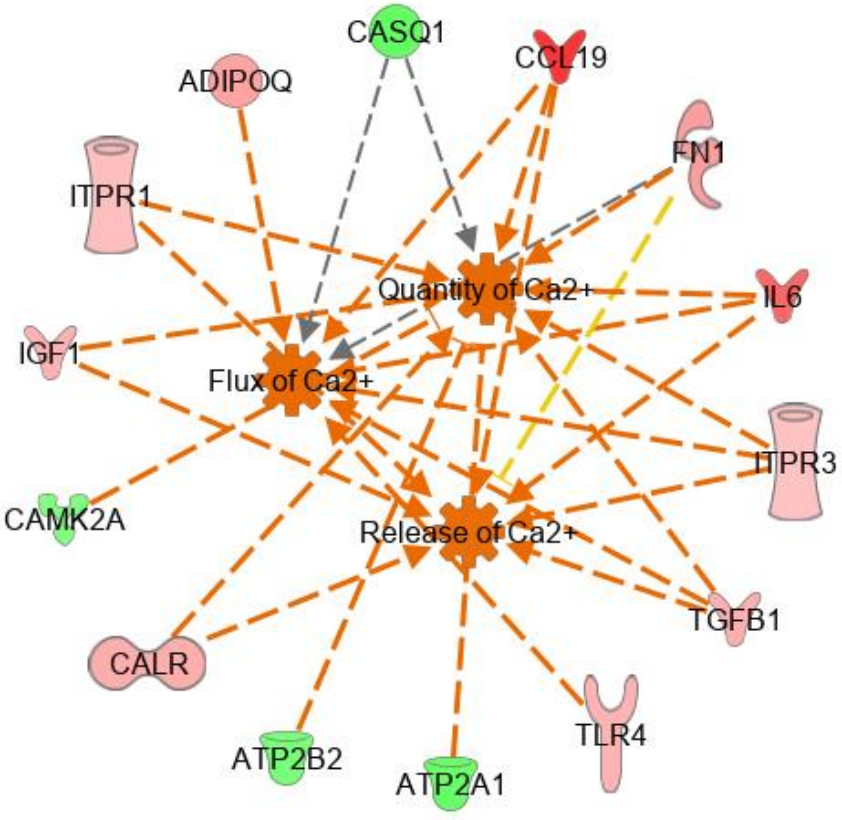
Cellular compromise



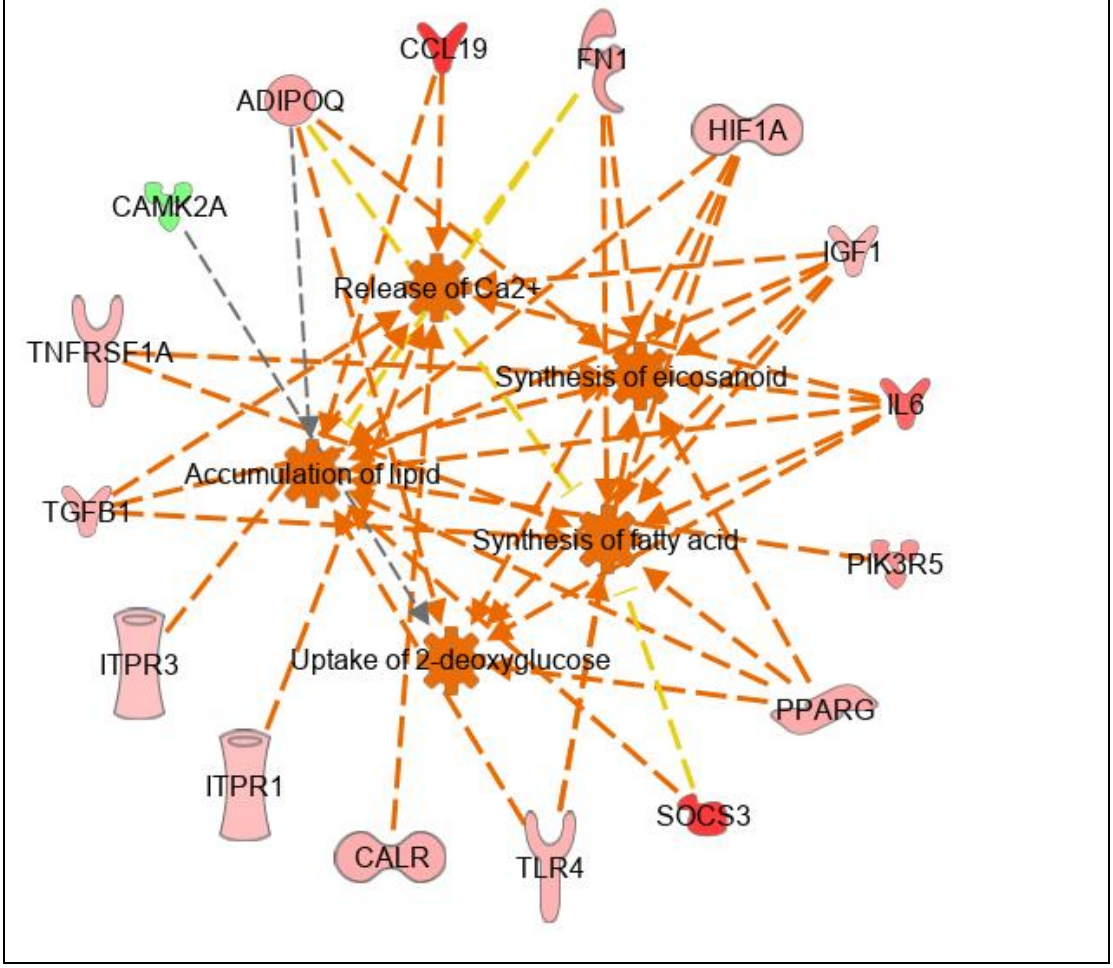
Molecular transport



Cell signalling



Small molecule biochemistry



9.2.2.4 Differentially expressed genes in both the control and fractured limb in CFS 5, 6 and7 using CFS 4 as the comparator

Control Limb			
CFS 5 with CFS 4			
Symbol	Entrez Gene Name	Expr Log Ratio	Expr p-value
FABP3	fatty acid binding protein 3	-0.896	0.0254
MYL1	myosin light chain 1	-0.802	0.0319
CFS 6 with CFS 4			
Symbol	Entrez Gene Name	Expr Log Ratio	Expr p-value
ADIPOQ	adiponectin, C1Q and collagen domain containing	1.484	0.0301
FOXO1	forkhead box O1	0.686	0.0322
MAP1LC3A	microtubule associated protein 1 light chain 3 alpha	2.338	0.0418
PPARG	peroxisome proliferator activated receptor gamma	0.796	0.0144
CFS 7 with CFS 4			
Symbol	Entrez Gene Name	Expr Log Ratio	Expr p-value
ATP2A1	ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 1	-0.83	0.0136
CASQ1	calsequestrin 1	-0.599	0.0086
FOXO1	forkhead box O1	1.153	0.000318
FOXO3B	forkhead box O3B	0.634	0.0041
FST	follistatin	0.84	0.0012
ITPR3	inositol 1,4,5-trisphosphate receptor type 3	0.849	0.0136
MYH2	myosin heavy chain 2	-1.017	0.0049
MYL1	myosin light chain 1	-1.054	0.00001
OSTN	osteocrin	-1.311	0.0021
PDK4	pyruvate dehydrogenase kinase 4	0.762	0.0174
PKM	pyruvate kinase M1/2	-0.684	0.000171

SREBF1	sterol regulatory element binding transcription factor 1	0.804	0.0373

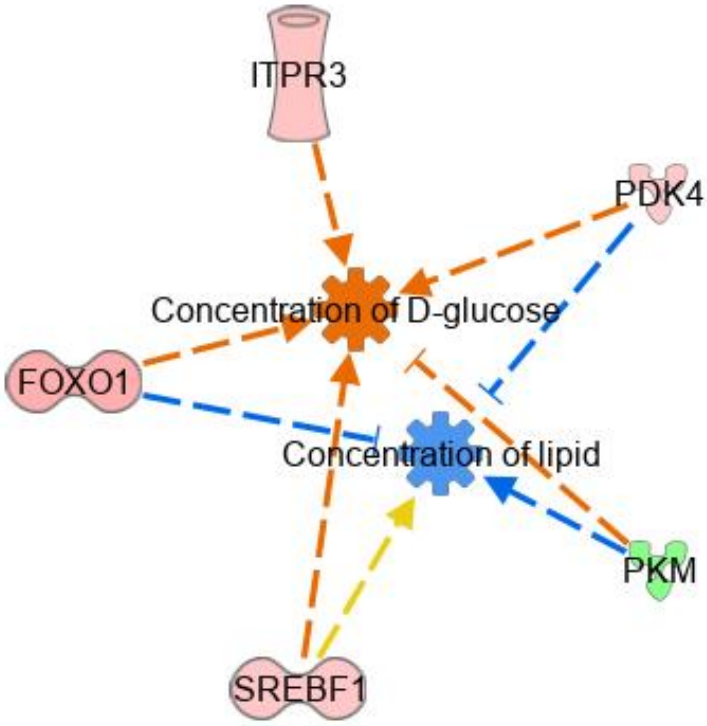
Fractured Limb			
CFS 5 (n=20) with CFS 4 (n=19)			
Symbol	Entrez Gene Name	Expr Log Ratio	Expr p-value
CALR	calreticulin	0.796	0.0303
CANX	calnexin	0.658	0.0121
COL1A1	collagen type I alpha 1 chain	1.219	0.0193
COL6A3	collagen type VI alpha 3 chain	0.708	0.0308
CTSL	cathepsin L	0.874	0.0411
HIF1A	hypoxia inducible factor 1 subunit alpha	0.71	0.049
IGF1	insulin like growth factor 1	0.77	0.0499
MSTN	myostatin	-1.139	0.0103
MYH4	myosin heavy chain 4	-0.631	0.0441
PPARG	peroxisome proliferator activated receptor gamma	0.668	0.0397
SOD2	superoxide dismutase 2	0.958	0.0184
TGFB1	transforming growth factor beta 1	0.615	0.0413
CFS 6 (n=19) with CFS 4 (n=19)			
Symbol	Entrez Gene Name	Expr Log Ratio	Expr p-value
ADIPOQ	adiponectin, C1Q and collagen domain containing	1.593	0.0392
CALR	calreticulin	0.635	0.0335
CANX	calnexin	0.619	0.0177
CASP8	caspase 8	0.724	0.0351
CCL19	C-C motif chemokine ligand 19	2.033	0.0192
COL1A1	collagen type I alpha 1 chain	1.513	0.004
COL4A1	collagen type IV alpha 1 chain	0.724	0.0403
CTSL	cathepsin L	0.747	0.0381
IGFBP1	insulin like growth factor binding protein 1	1.465	0.0109

ITPR3	inositol 1,4,5-trisphosphate receptor type 3	1.399	0.0018
MSTN	myostatin	-0.984	0.0193
MYL6	myosin light chain 6	0.779	0.0238
NAT1	N-acetyltransferase 1	1.384	0.0179
PIK3R5	phosphoinositide-3-kinase regulatory subunit 5	0.931	0.038
PPARG	peroxisome proliferator activated receptor gamma	1.098	0.0048
TGFB1	transforming growth factor beta 1	0.655	0.0333
TRAF3	TNF receptor associated factor 3	0.662	0.0128
CFS 7 (n=19) with CFS 4 (n=19)			
Symbol	Entrez Gene Name	Expr Log Ratio	Expr p-value
ATP2A1	ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 1	-1.067	0.033
ATP6AP2	ATPase H+ transporting accessory protein 2	0.595	0.012
CALR	calreticulin	0.733	0.011
CANX	calnexin	0.773	0.001
CAPN2	calpain 2	0.78	0.000
CASP8	caspase 8	0.58	0.035
CASQ1	calsequestrin 1	-0.78	0.037
CCL19	C-C motif chemokine ligand 19	2.258	0.005
COL1A1	collagen type I alpha 1 chain	1.338	0.007
COL6A3	collagen type VI alpha 3 chain	0.819	0.004
CTSL	cathepsin L	0.827	0.021
FOXO1	forkhead box O1	0.734	0.024
FST	follistatin	0.921	0.008
IFI30	IFI30 lysosomal thiol reductase	1.17	0.020
IGF1	insulin like growth factor 1	0.838	0.012
ITPR3	inositol 1,4,5-trisphosphate receptor type 3	1.173	0.000
MSTN	myostatin	-1.091	0.003
MTFR1	mitochondrial fission regulator 1	0.618	0.001

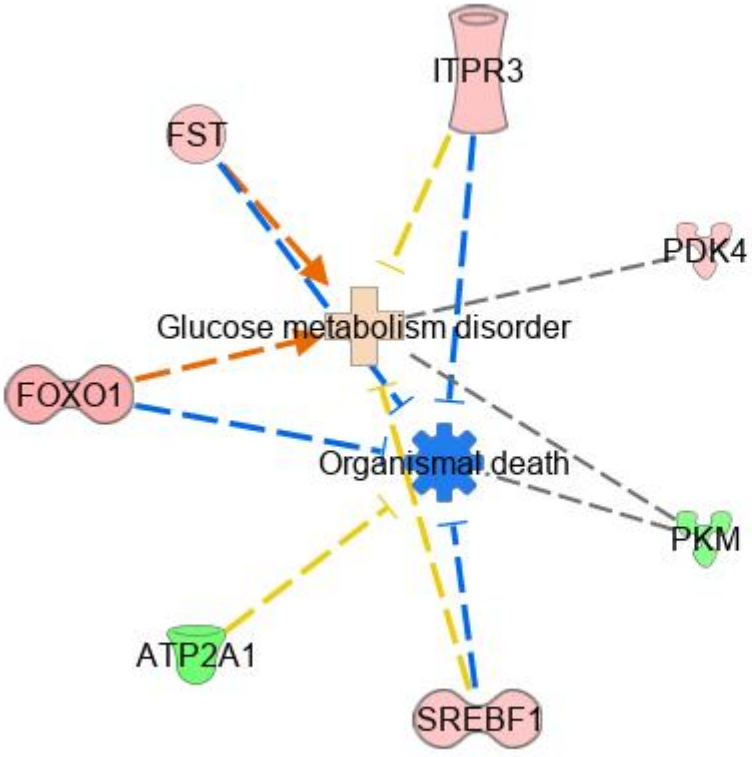
MYF5	myogenic factor 5	0.645	0.037
MYH2	myosin heavy chain 2	-0.974	0.023
MYL1	myosin light chain 1	-0.846	0.024
MYLK2	myosin light chain kinase 2	-1.097	0.006
PDK2	pyruvate dehydrogenase kinase 2	-0.667	0.005
PIK3R5	phosphoinositide-3-kinase regulatory subunit 5	0.791	0.046
PPARG	peroxisome proliferator activated receptor gamma	0.922	0.008
PSMA1	proteasome 20S subunit alpha 1	0.603	0.000
PSMC4	proteasome 26S subunit, ATPase 4	0.666	0.003
PYGM	glycogen phosphorylase, muscle associated	-0.663	0.044
RHOA	ras homolog family member A	0.617	0.001
SOCS3	suppressor of cytokine signaling 3	1.229	0.035
SOD2	superoxide dismutase 2	1.976	0.000
TNC	tenascin C	2.604	0.002
TRAF3	TNF receptor associated factor 3	0.731	0.006
TRIM63	tripartite motif containing 63	0.916	0.018

9.2.2.5 Gene expression rosettes predicted by IPA in the non-fractured limb when comparing CFS 7 with CFS 4

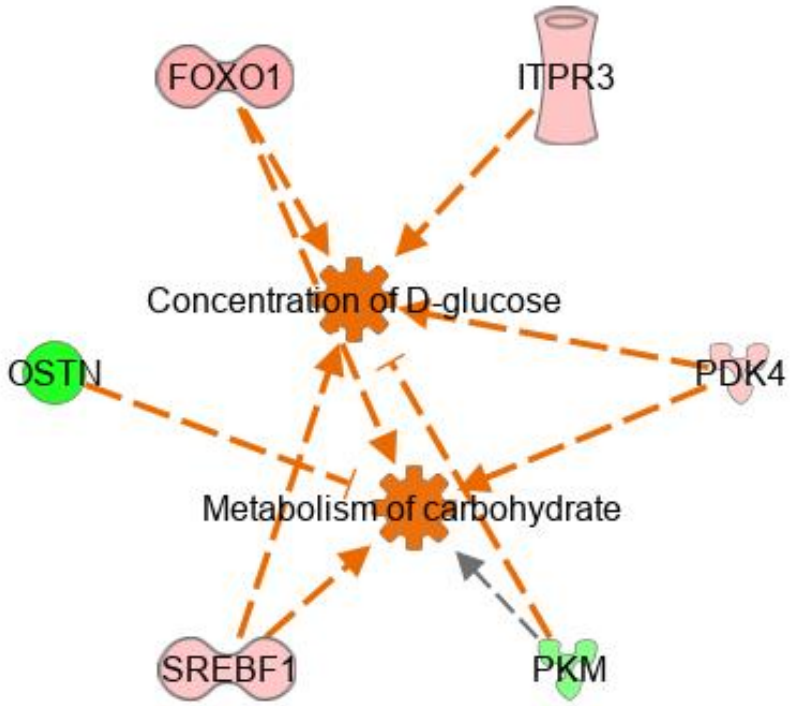
Molecular transport



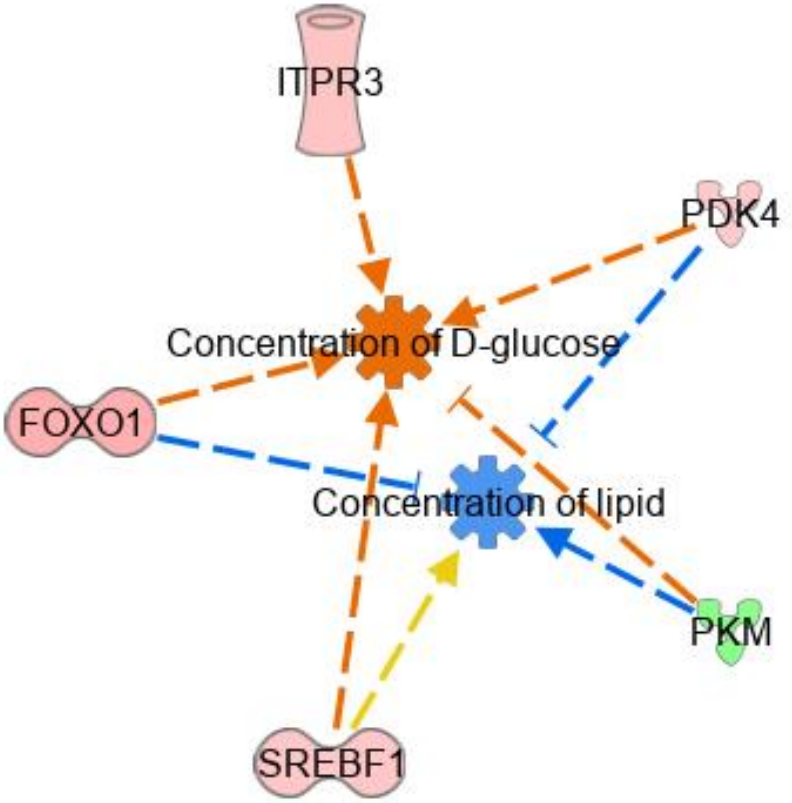
Organismal injury



Carbohydrate metabolism

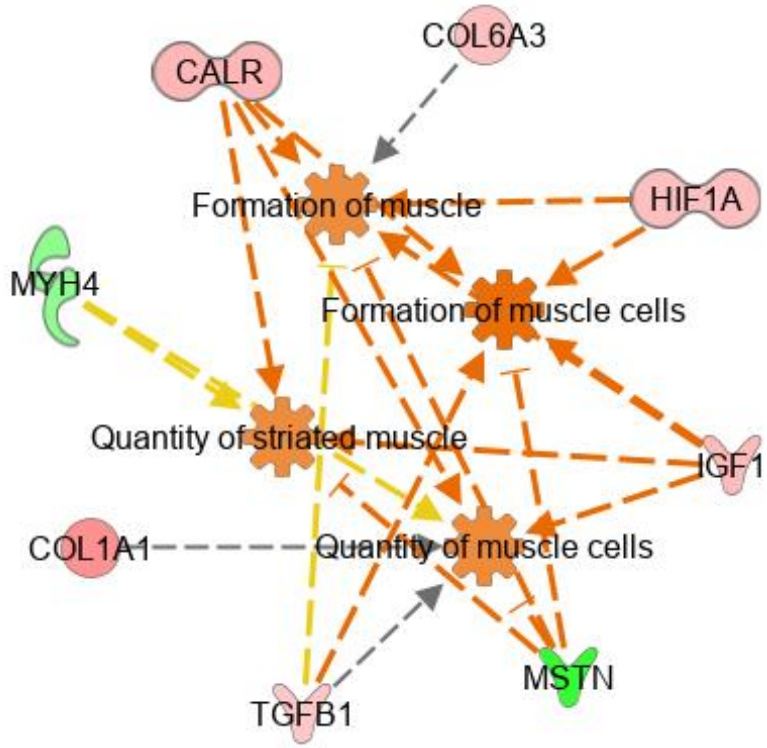


Small molecule biochemistry

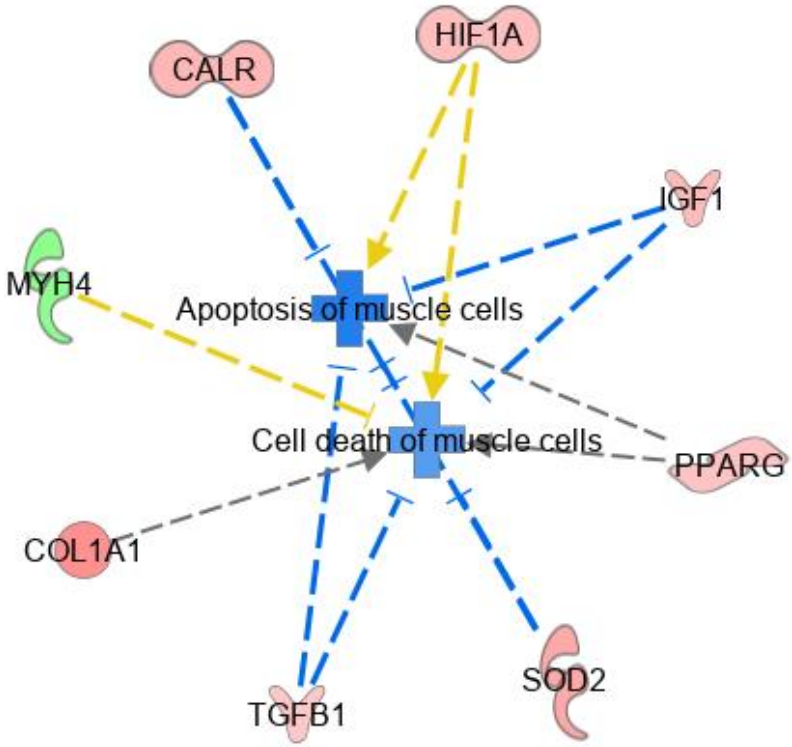


9.2.2.6 Gene expression rosettes predicted by IPA in the fractured limb when comparing CFS 5 with CFS 4

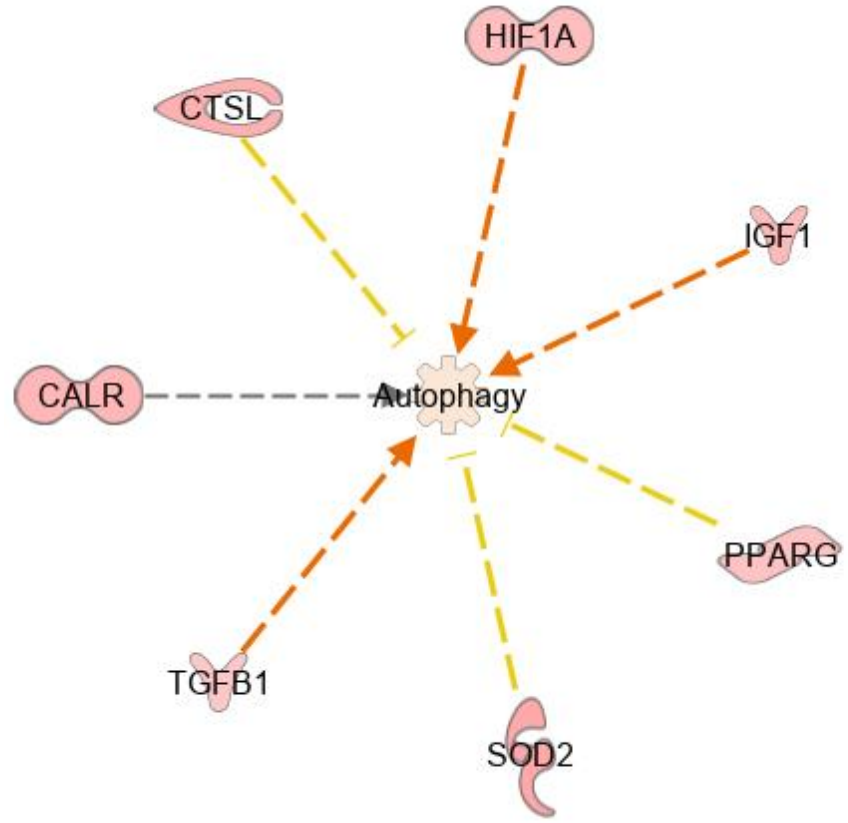
Skeletal and muscular system development



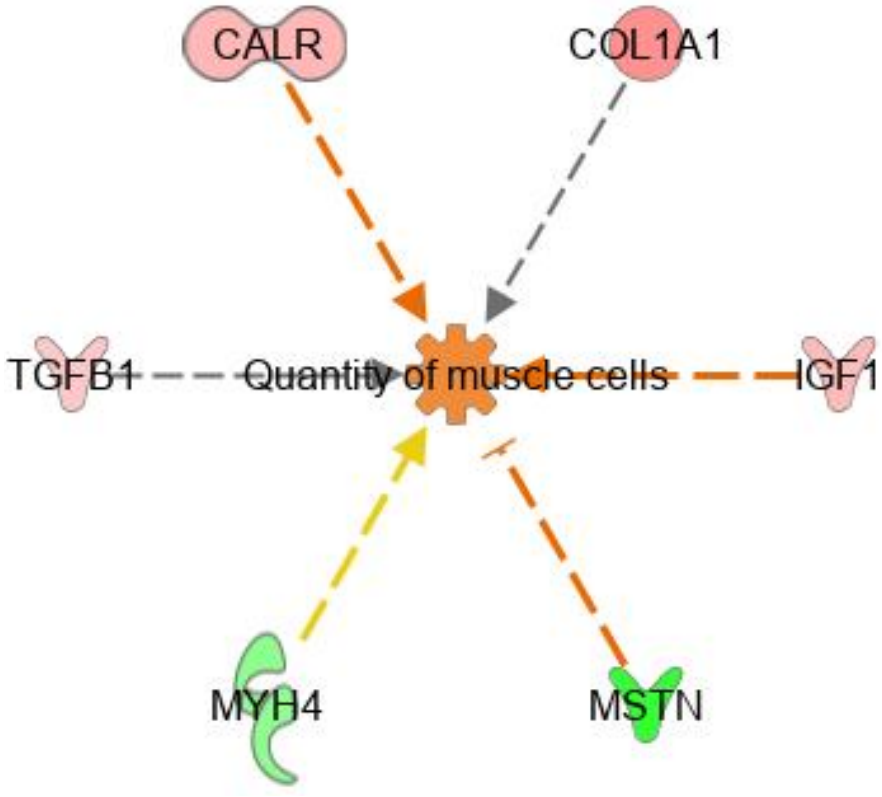
Cell death and survival



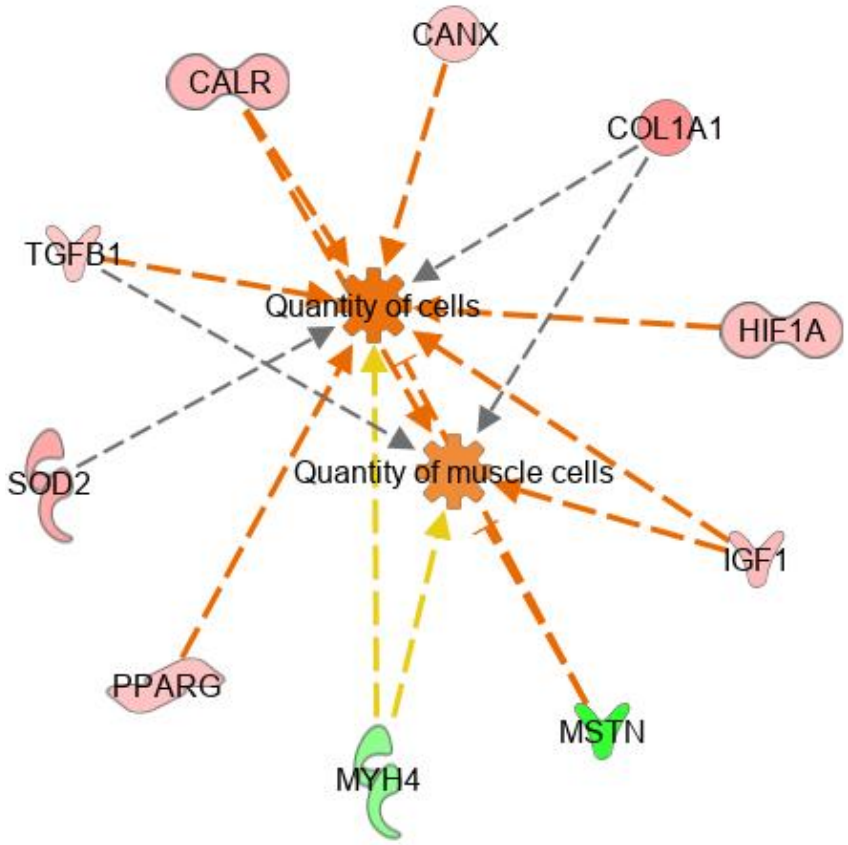
Cell morphology



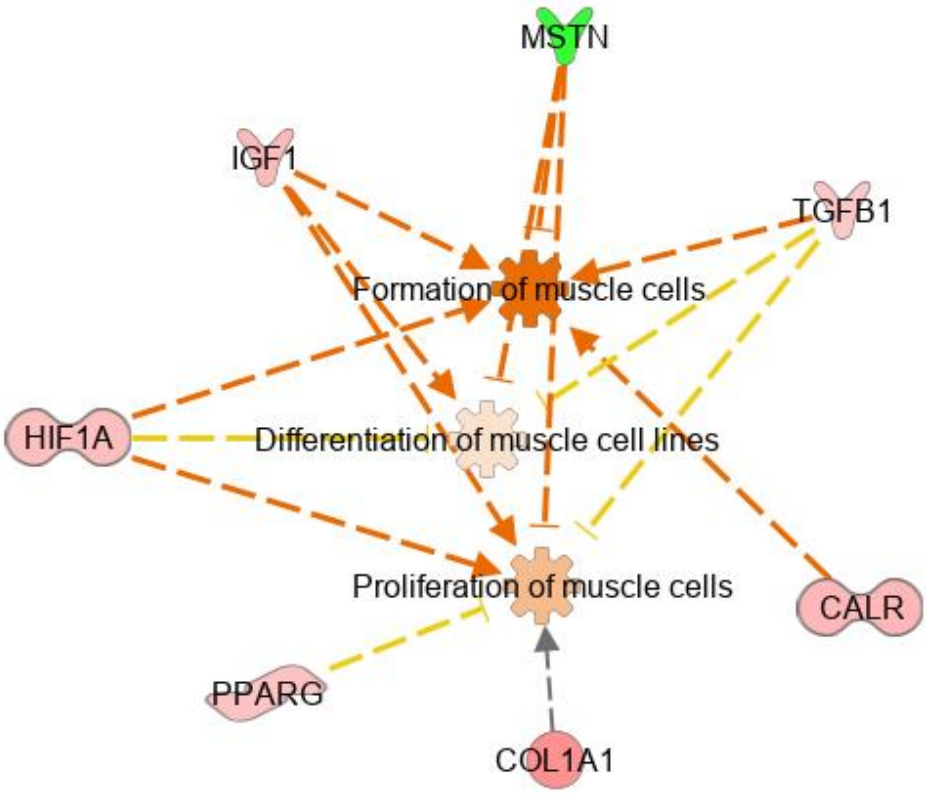
Organ morphology



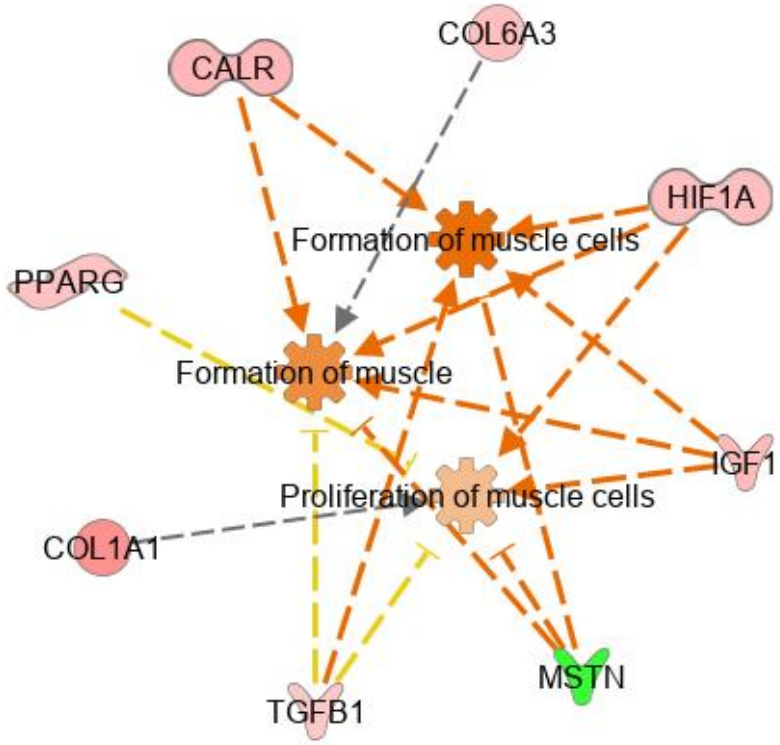
Tissue morphology



Cellular development

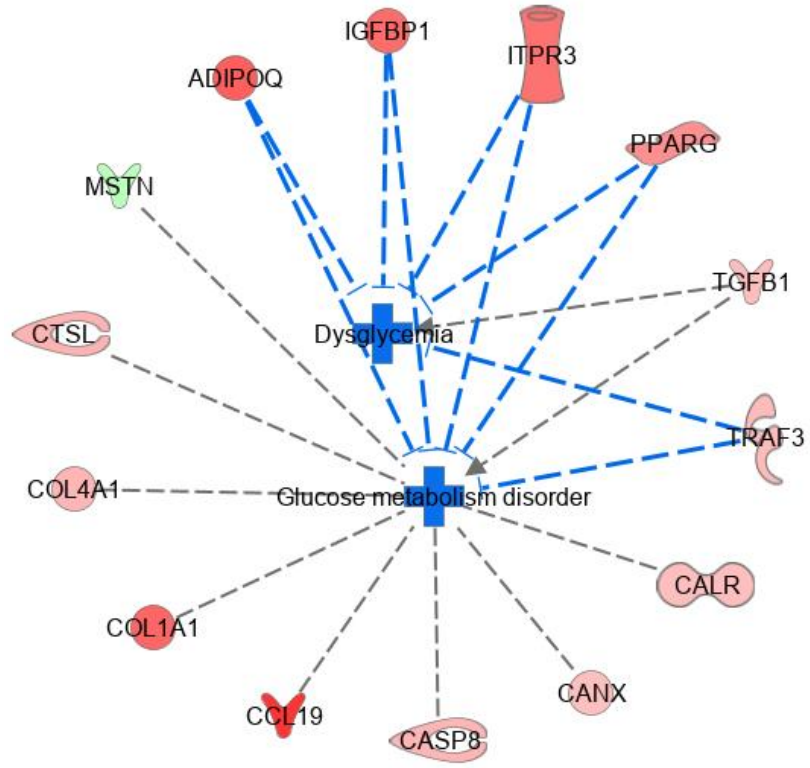


Tissue development

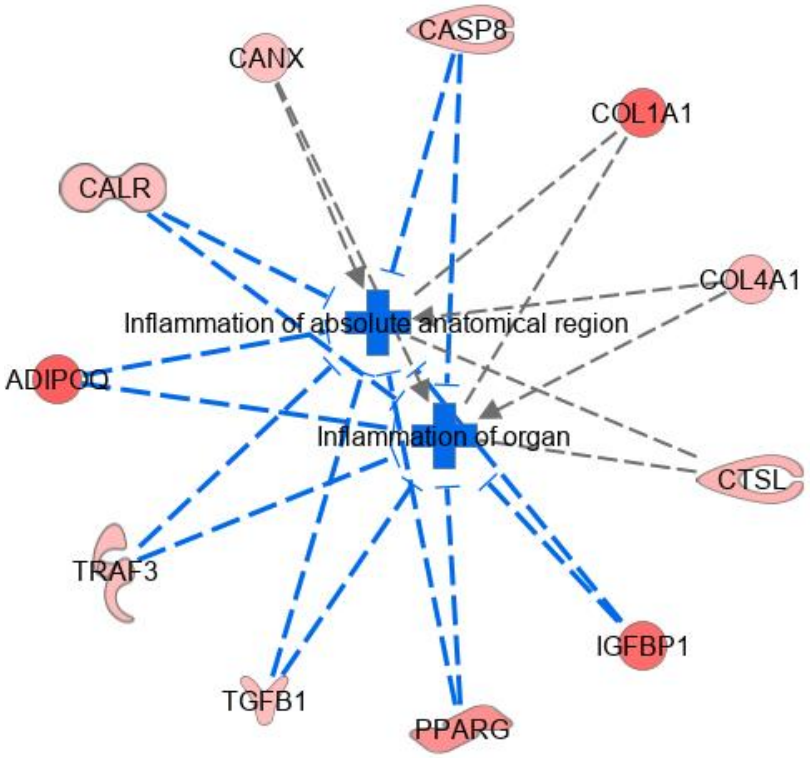


9.2.2.7 Gene expression rosettes predicted by IPA in the fractured limb when comparing CFS 6 with CFS 4

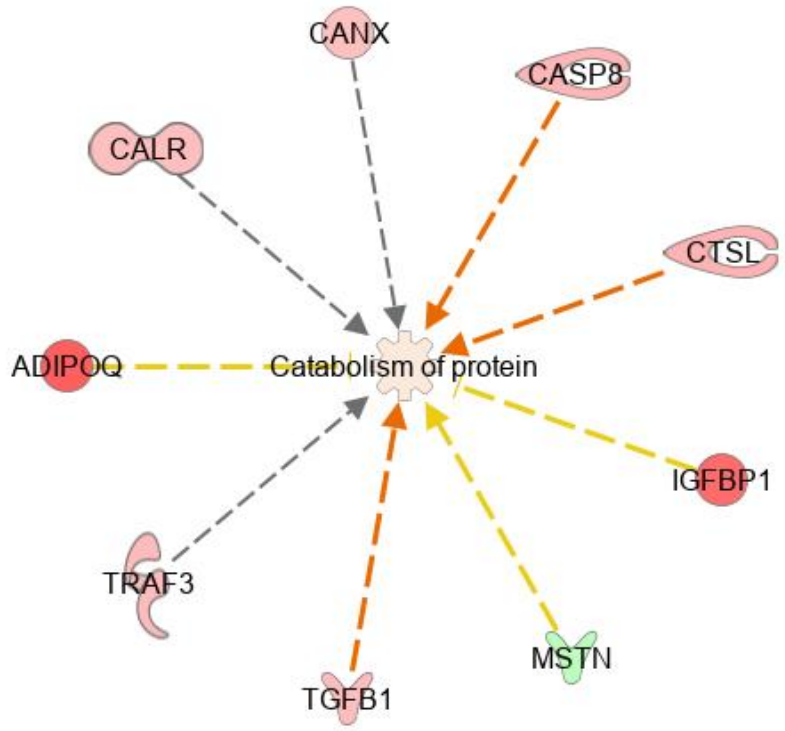
Metabolic disease



Inflammatory response

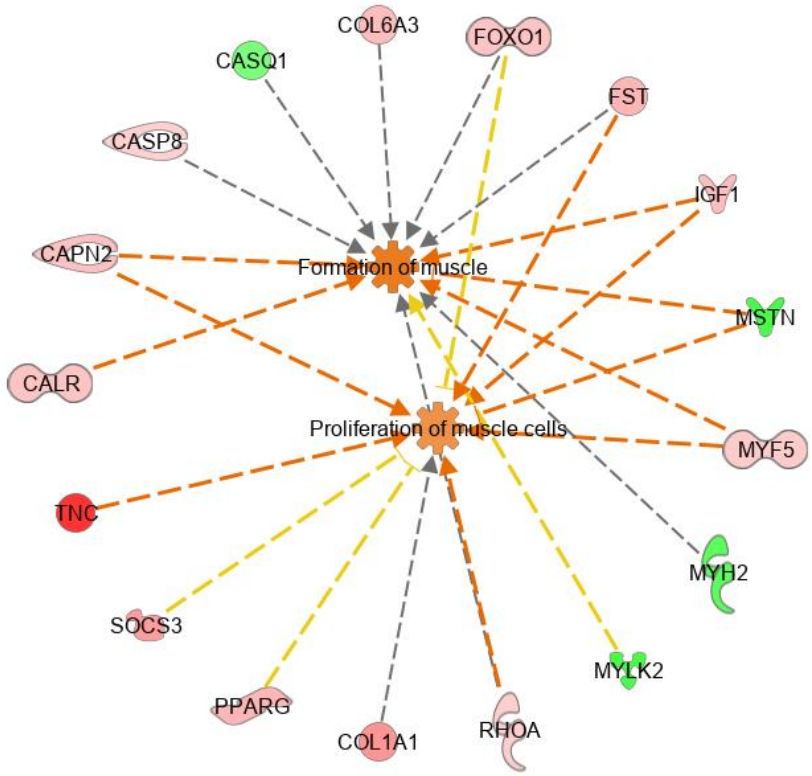


Protein degradation

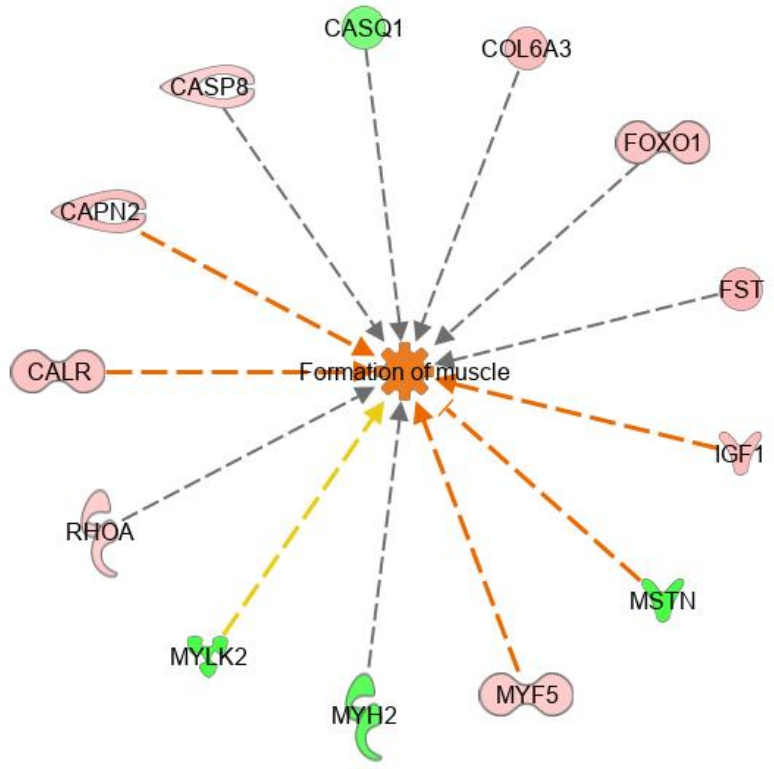


9.2.2.8 Gene expression rosettes predicted by IPA in the fractured limb when comparing CFS 7 with CFS 4

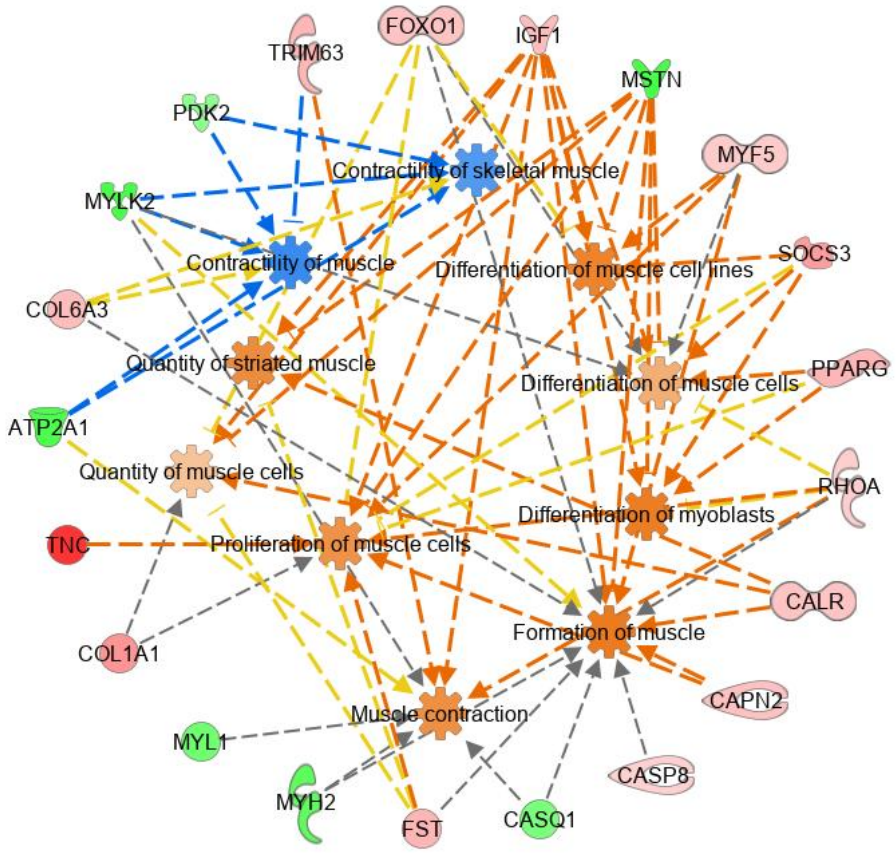
Organ development



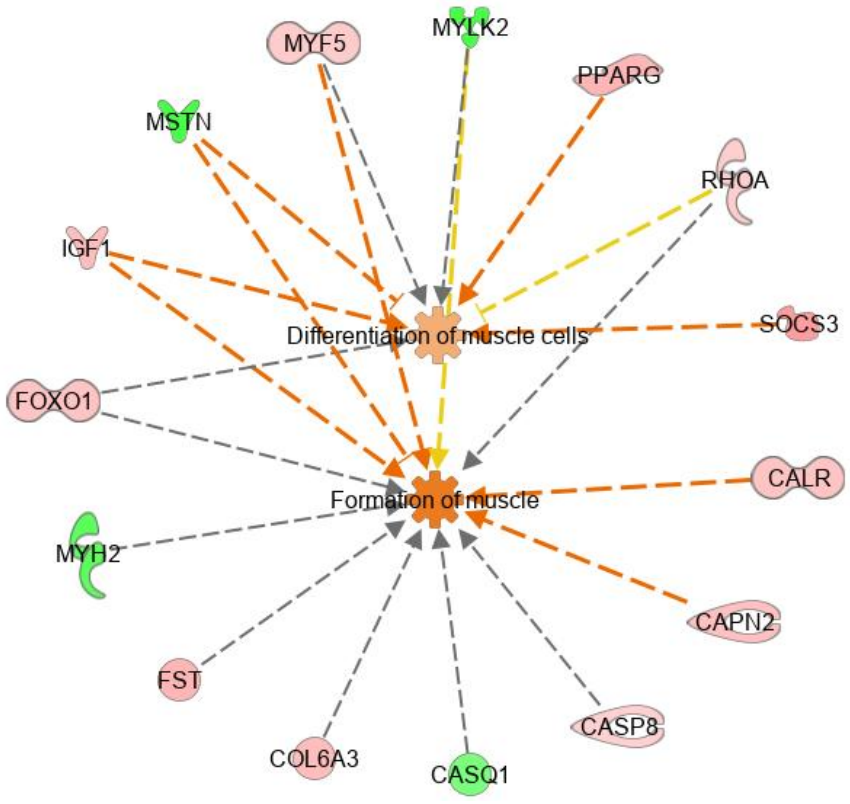
Organismal development



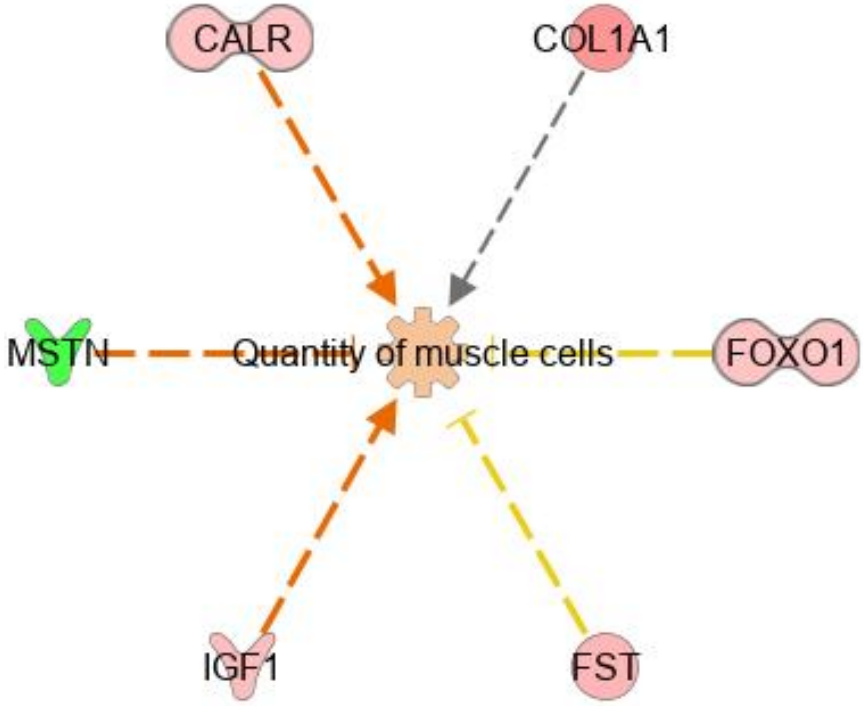
Skeletal and muscular development



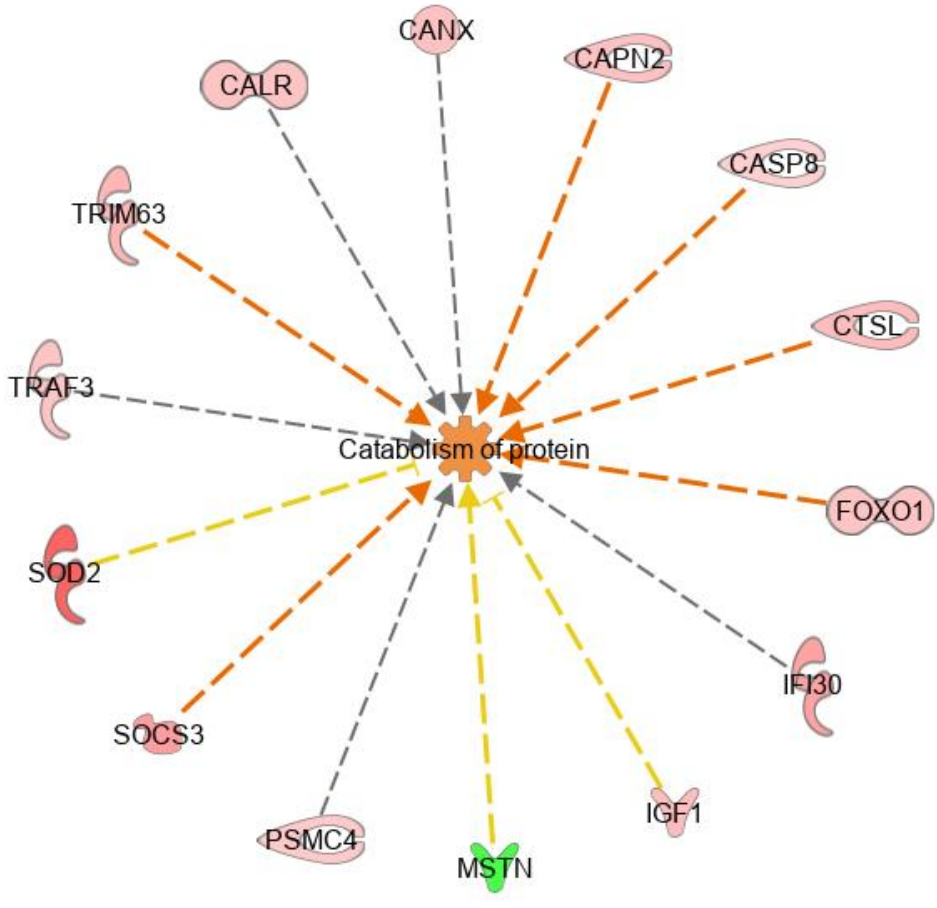
Tissue development



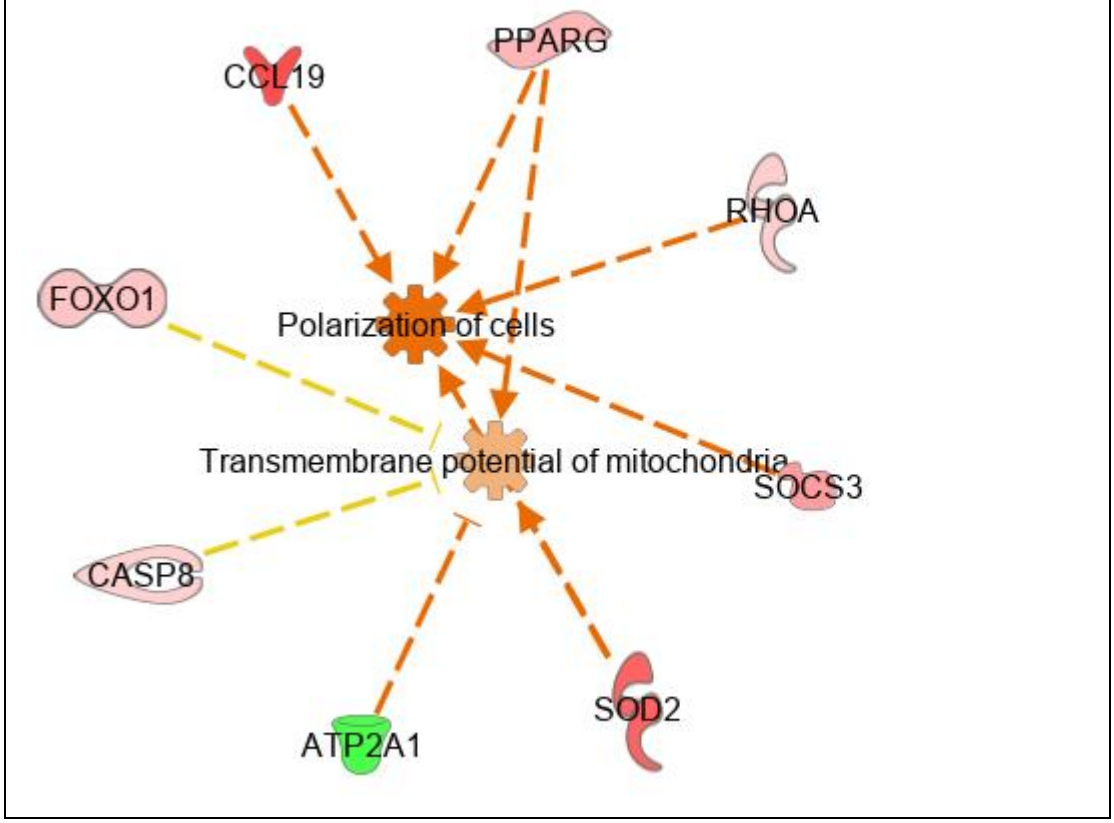
Organ morphology



Protein degradation



Cell morphology



9.2.2.9 FEMUR genes T-test results

Gene	Gene function	Fold change	p-value
TNC	tenascin C	22.90	0.00
CCL19	chemokine (C-C motif) ligand 19	20.74	0.03
MT1A	metallothionein 1A	17.46	0.01
SOCS3	suppressor of cytokine signaling 3	9.69	0.00
IL6	interleukin 6 (interferon, beta 2)	9.16	0.78
ADIPOQ	adiponectin, C1Q and collagen domain containing	6.09	0.00
IFI30	IFI30, lysosomal thiol reductase	5.86	0.00
COL1A1	collagen, type I, alpha 1	5.46	0.00
PPARGC1A	peroxisome proliferator-activated receptor gamma	3.40	0.69
HK2	hexokinase 2	3.31	0.86
SOD2	superoxide dismutase 2, mitochondrial	3.31	0.00
FN1	forkhead box O1	3.30	0.04
PIK3R5	phosphoinositide-3-kinase regulatory subunit 5	3.20	0.00
CTSL	alpha catenin	3.02	0.00
HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	2.56	0.71
IGF1	insulin-like growth factor 1 (somatomedin C)	2.54	0.80
ATG12	autophagy related 12	2.50	0.00
CALR	calreticulin	2.42	0.00
COL6A3	collagen type VI alpha 3 chain	2.40	0.44
ITPR3	inositol 1,4,5-trisphosphate receptor type 3	2.36	0.00
FGF21	fibronectin 1	2.30	0.00
CAMK4	calcium/calmodulin dependent protein kinase IV	2.21	0.00

Gene	Gene function	Fold change	p-value
TGFB1	transforming growth factor, beta 1	2.14	0.00
CASP8	caspase 8, apoptosis-related cysteine peptidase	2.01	0.00
MYL6	myosin, light chain 6, alkali, smooth muscle and non-muscle	1.98	0.00
TLR4	toll-like receptor 4	1.95	0.00
TNFRSF1A	TNF receptor superfamily member 1A	1.91	0.00
ITPR1	inositol 1,4,5-trisphosphate receptor type 1	1.86	0.82
COL4A1	collagen type IV alpha 1 chain	1.85	0.00
SCNN1A	sodium channel epithelial 1 alpha subunit	1.83	0.00
LDHA	lactate dehydrogenase A	1.79	0.00
TRAF3	TNF receptor-associated factor 3	1.68	0.03
CANX	calnexin	1.66	0.16
ATP2B4	ATPase plasma membrane Ca2+ transporting 4	1.62	0.00
ATP6AP2	ATPase H+ transporting accessory protein 2	1.61	0.00
ACO1	aconitase 1	1.53	0.00
B2M	beta-2-microglobulin	1.51	0.00
GLUD1	glutamate dehydrogenase 1	1.49	0.96
PDIA2	protein disulfide isomerase family A member 2	1.49	0.28
CPT1A	carnitine palmitoyltransferase 1A (liver)	1.47	0.43
TJP2	tight junction protein 2	1.47	0.00
STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)	1.46	0.00
MYH1	myosin heavy chain 1	1.45	0.01
BECN1	beclin 1, autophagy related	1.45	0.31
CYCS	cullin 4A	1.44	0.37
MAPK3	mitogen-activated protein kinase 3	1.42	0.00

Gene	Gene function	Fold change	p-value
PLIN3	perilipn 3	1.37	0.76
CASP3	caspase 3	1.36	0.00
AKT1	v-akt murine thymoma viral oncogene homolog 1	1.32	0.68
UQCC2	Ubiquinol-Cytochrome C Reductase Complex Assembly Factor 2	1.32	0.58
CALM1	calmodulin 1	1.31	0.00
TRIM63	tripartite motif containing 63, E3 ubiquitin protein ligase	1.31	0.31
MCUR1	mitochondrial calcium uniporter regulator 1	1.30	0.52
CAPN2	calpain 2, (m/II) large subunit	1.30	0.08
STAT1	signal transducer and activator of transcription 1, 91kDa	1.29	0.00
SLC39A6	Solute Carrier Family 39 Member 6	1.29	0.80
IL6R	interleukin 6 receptor	1.28	0.46
NFE2L2	nuclear factor, erythroid 2-like 2	1.27	0.60
ATF6	activating transcription factor 6	1.27	0.00
IKBKB	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	1.27	0.02
MYH3	myosin, heavy chain 3, skeletal muscle, embryonic	1.26	0.00
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	1.26	0.48
OSTN	osteocrin	1.25	0.63
RHOA	ras homolog family member A	1.25	0.86
JKAMP	JNK1/MAPK8-associated membrane protein	1.25	0.25
SREBF1	sterol regulatory element binding transcription factor 1	1.25	1.00
PTEN	phosphatase and tensin homolog	1.24	0.00
MTFR1	mitochondrial fission regulator 1	1.23	0.61
FABP3	F-box protein 32	1.23	0.00
ATF4	activating transcription factor 4	1.23	0.00

Gene	Gene function	Fold change	p-value
TFAM	transcription factor A, mitochondrial	1.22	0.01
PSMC4	proteasome 26S subunit, ATPase 4	1.22	0.98
FST	follistatin	1.22	0.00
EIF2S1	dihydrolipoamide dehydrogenase	1.22	0.52
PSMA1	proteasome (prosome, macropain) subunit, alpha type, 1	1.21	0.00
BRD4	bromodomain containing 4	1.21	0.50
CAPN1	calpain 1, (mu/I) large subunit	1.20	0.00
EIF4E	eukaryotic translation initiation factor 4E	1.18	0.33
TFB2M	transcription factor B2, mitochondrial	1.17	0.00
CRTC1	carnitine palmitoyltransferase 1B (muscle),CHKB-CPT1B readthrough (NMD candidate)	1.17	0.36
COX5A	cytochrome c oxidase subunit 5A	1.15	0.00
IDH2	isocitrate dehydrogenase (NADP(+)) 2, mitochondrial	1.15	0.00
PKM	pyruvate kinase, muscle	1.14	0.00
PRKAB1	protein kinase AMP-activated non-catalytic subunit beta 1	1.14	0.00
EIF4EBP1	eukaryotic translation initiation factor 4B	1.14	0.05
IRS1	insulin receptor substrate 1	1.13	0.00
CAT	catalase	1.13	0.00
PRKAA1	protein kinase, AMP-activated, alpha 1 catalytic subunit	1.13	0.67
RELA	RELA proto-oncogene, NF-kB subunit	1.12	0.00
FBXO32	fibroblast growth factor 21	1.12	0.97
SMAD2	SMAD family member 2	1.12	0.61
PSMC1	proteasome (prosome, macropain) 26S subunit, ATPase, 1	1.12	0.01
PDHA1	pyruvate dehydrogenase (lipoamide) alpha 1	1.11	0.00
MYO5C	myosin VC	1.11	0.00

Gene	Gene function	Fold change	p-value
MTOR	mechanistic target of rapamycin (serine/threonine kinase)	1.10	0.00
FOXO1	forkhead box O3B pseudogene,forkhead box O3	1.10	0.03
PPP3CA	protein phosphatase 3 catalytic subunit alpha	1.10	0.00
DLD	dihydrolipoamide S-acetyltransferase	1.10	0.03
ALDH2	aldehyde dehydrogenase 2 family (mitochondrial)	1.10	0.45
EIF4E	eukaryotic translation initiation factor 4E	1.10	0.09
MYF6	myogenic factor 6	1.10	0.00
KCNT1	potassium sodium-activated channel subfamily T member 1	1.10	0.16
PPARG	peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	1.09	0.06
PAX7	paired box 7	1.09	0.04
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	1.08	0.00
MPC2	mitochondrial pyruvate carrier 2	1.08	0.00
ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	1.07	0.00
SRF	serum response factor	1.07	0.37
PDK4	pyruvate dehydrogenase kinase, isozyme 4	1.07	0.22
ATP5I	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit E	1.07	0.00
TRPM7	transient receptor potential cation channel subfamily M member 7	1.07	0.28
MDH2	malate dehydrogenase 2	1.07	0.25
CUL4A	cathepsin L	1.07	0.00
MYH4	myosin, heavy chain 4, skeletal muscle	1.06	0.00
TUFM	Tu translation elongation factor, mitochondrial	1.06	0.00
DLAT	cytochrome c, somatic	1.06	0.22
HADH	hydroxyacyl-CoA dehydrogenase	1.05	0.00
RPS6KB2	ribosomal protein S6 kinase, 70kDa, polypeptide 2	1.05	0.08

Gene	Gene function	Fold change	p-value
ATG9A	autophagy related 9A	1.05	0.00
MAPK9	mitogen-activated protein kinase 9	1.04	0.00
CARM1	coactivator associated arginine methyltransferase 1	1.04	0.21
MYL1	myosin, light chain 1, alkali; skeletal, fast	1.04	0.52
COX6B1	cytochrome c oxidase subunit 6B1	1.03	0.57
CASQ2	calsequestrin 2	1.03	0.00
IGBP1	immunoglobulin (CD79A) binding protein 1	1.03	0.04
ZFAND5	zinc finger AN1-type containing 5	1.03	0.25
ATP2A2	ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 2	1.03	0.01
POLRMT	polymerase (RNA) mitochondrial (DNA directed)	1.03	0.00
GSK3B	glycogen synthase kinase 3 beta	1.02	0.19
MYOG	myogenin (myogenic factor 4)	1.02	0.00
CTNNAL1	citrate synthase	1.02	0.44
SDHA	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	1.02	0.00
CS	CREB regulated transcription coactivator 1	1.01	0.13
COX5B	cytochrome c oxidase subunit Vb	1.01	0.00
GPD2	glycerol-3-phosphate dehydrogenase 2 (mitochondrial)	1.01	0.08
OGDH	oxoglutarate dehydrogenase	1.01	0.06
RPS6KB1	ribosomal protein S6 kinase B1	1.01	0.00
PDPK1	3-phosphoinositide-dependent protein kinase 1	1.00	0.03
NDUFS6	NADH:ubiquinone oxidoreductase subunit B6	1.00	0.00
EIF4B	eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa	1.00	0.36
MYF5	myogenic factor 5	0.99	0.02
TNNT1	troponin T1, slow skeletal type	0.99	0.00

Gene	Gene function	Fold change	p-value
ACTA1	actin, alpha 1, skeletal muscle	0.99	0.00
NDUFB5	NADH:ubiquinone oxidoreductase subunit B5	0.99	0.08
RPTOR	regulatory associated protein of MTOR, complex 1	0.98	0.00
STIM1	stromal interaction molecule 1	0.97	0.04
MSTN	myostatin	0.97	0.48
ATP2B2	ATPase plasma membrane Ca2+ transporting 2	0.97	0.75
RICTOR	RPTOR independent companion of MTOR, complex 2	0.96	0.07
FOXO3B	fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)	0.96	0.00
TTN	titin	0.95	0.18
NDUFB3	NADH:ubiquinone oxidoreductase subunit B3	0.95	0.04
HSPA8	heat shock 70kDa protein 8	0.95	0.00
PPARA	peroxisome proliferator-activated receptor alpha	0.94	0.00
INSR	insulin receptor	0.93	0.00
NRF1	nuclear respiratory factor 1	0.93	0.05
MPST	mercaptopyruvate sulfurtransferase	0.93	0.00
ATP2A1	ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 1	0.93	0.00
MYH2	myosin heavy chain 2	0.92	0.68
PFKM	phosphofructokinase, muscle	0.91	0.43
PDK2	pyruvate dehydrogenase kinase, isozyme 2	0.90	0.00
GYS1	glycogen synthase 1	0.90	0.00
OPTN	optineurin	0.89	0.07
UCP3	uncoupling protein 3 (mitochondrial, proton carrier)	0.89	0.20
CPT1B	eukaryotic translation initiation factor 4E binding protein 1	0.89	0.09
PAX3	paired box 3	0.88	0.00

Gene	Gene function	Fold change	p-value
TNNC2	troponin C2, fast skeletal type	0.88	0.00
PRKAA2	protein kinase, AMP-activated, alpha 2 catalytic subunit	0.87	0.00
MYBPC1	myosin binding protein C, slow type	0.87	0.00
MYLPF	myosin light chain, phosphorylatable, fast skeletal muscle	0.86	0.00
RYR1	ryanodine receptor 1	0.86	0.00
PARK2	parkin RBR E3 ubiquitin protein ligase	0.85	0.03
SIX1	SIX homeobox 1	0.85	0.00
PYGM	phosphorylase, glycogen, muscle	0.84	0.80
SLC2A4	solute carrier family 2 member 4/Glut4 transporter	0.84	0.35
AMPD1	adenosine monophosphate deaminase 1	0.83	0.05
JPH1	junctophilin 1	0.82	0.48
CAMK2A	calcium/calmodulin dependent protein kinase II alpha	0.81	0.01
CASQ1	calsequestrin 1	0.79	0.12
MYLK2	myosin light chain kinase 2	0.72	0.28

9.3 Appendices C - Inflammatory markers and outcomes following hip fracture

9.3.1 FEMUR study preparation of serum samples V1.1

FEMUR STUDY	Standard Operating Procedure
Title: Preparation of Serum Samples	
Reference: SOP-Serum Prep Version: 1.1 Date: 08/07/2024	Effective From: Commencement of Femur study Review Cycle: 2 Years from Date
Author: Tony Kelly, Waheed Ashraf Authorised By: BJO	
Document History: 1. 1.0 – 31/10/2019 2. 1.1 – 22/01/2020	Modification to Previous Version: 1. New Standard Formatting

1. Document History

Version Number	Effective Date	Reason for Change
1.1	22/01/2020	New Standard Formatting

2. Introduction

This document outlines guidelines for managing blood samples prior to processing and the separation of serum from whole blood using a centrifuge.

3. Purpose and Scope

This SOP is created to ensure that the procedure is followed for managing participant blood samples and preparing serum by means of centrifugation. It applies to staff using the clinical research facilities.

- All members of staff are responsible for following this procedure.

4. Responsibilities

It is the responsibility of all clinical staff to ensure they follow the guidance in this SOP.

5. Definitions

SOP: Standard operating Procedure - Document that sets out the scope, roles, responsibilities and the method that must be followed

Sample: Any biological tissue taken from a research participant

Whole blood samples collected for Femur study:

Bloods are to be collected and processed for the following samples:

- **RNA:** 2.5ml, PAXgene Vacutainer tube (Clear with red lid). Left to stand for 2 hours, prior to being frozen at -20^{o}C (this prevents damage to PET tube)
- **DNA:** 4ml, Purple Vacutainer tube. No standing time required, frozen at -20^{o}C
- **Serum:** 6ml, Gold Vacutainer serum separation tube. Stand for 30 minutes, then process according to procedure detailed here.

All tubes to be stored should be labelled with participant ID and date collected. Accurate records will be maintained of all stored samples in the TEAMS folder – **samples log.**

6. Procedure

Items Required

- Centrifuge with secondary containment of samples
- Gloves
- Sharps Bin
- Tray to hold blood tube(s)
- 6 x 2ml micro tubes and lids - sufficient for two Serum Separation Tubes (SST)
- Micro tube labels – printed using Dymo laboratory label printer
- Freezer-safe marker pen to number sample lids 1 – 6
- Suitable freezer storage boxes for whole blood and serum samples

Preparation of Serum should only be undertaken by qualified staff who have received training in doing so. Lab coat and gloves must be worn. Clinical staff taking samples should inform laboratory staff regarding any known issues associated with the sample.

Associated Hazards

- Blood Spillage

Emergency / Spillage Procedure

- Wear gloves
- Clean up spillage using approved laboratory disinfectant solution
- Dispose of paper towels, gloves etc. in clinical waste

Procedure for separation of serum

Bloods collected in 6ml Gold SST vacutainer tubes:

- Bloods are to be spun in the preparation area centrifuge using the following settings:
    - 3000rpm
    - 4^{o}C
    - 5 minutes

- Remove sample tube from centrifuge and place in tray.
- Carefully aliquot serum supernatant into 3 micro tubes, using a disposable Pasteur pipette. Ensure that the pellet is not disturbed. Repeat procedure for second SST
- Label tubes with participant ID number, project code and date as shown in example below
- Discard blood tube and pipette into sharps bin.

FEMUR Serum Pt. ID: 090 08/11/2021

Details are to be printed on tube labels in this format.

Sample Storage

- Samples will be stored in a labelled box, ordered by participant ID number.
- Boxes containing serum will be stored in the -80^{o}C freezer located in the research sample freezer and whole blood in the -20 prep room freezers.

Record Keeping

- Accurate records of stored samples are maintained on the Femur samples log located in the Femur TEAMS folder.

7. References and Associated Documents

N/A

9.3.2 Supplementary tables

9.3.2.1 Supplementary Table 8.3.2.1

	Hip fractures (frail)	Healthy controls	Multiple rib fractures (non-frail)	Prefrail controls
n	59	62	30	12

Mean Age Years (SD)	87.7 (5.9)	42 (11.57)	60.4 (14.2)	70.1 (7.16)
Sex M	9 (15%)	16 (26%)	21 (70%)	3 (26%)
F	50 (85%)	46 (74%)	9 (30%)	9 (74%)

Descriptive characteristics of hip fracture (FEMUR) cohort, health controls (PANTHER), multiple rib fractures (OPERA) cohort and Molecular Pathways Involved in Knee Pain: an Observational Study

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