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Sarcopenia in Older Women

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Abstract

Loss of skeletal muscle mass and function with age or 'sarcopenia' has attracted much interest due to its association with adverse outcomes, increased morbidity, and mortality. The discovery of pharmacological treatment options for sarcopenia remains in its infancy and is limited by incomplete knowledge of the complex interactions between different regulatory pathways in muscle homeostasis.

Efforts to harmonize a sarcopenia definition has resulted in a general agreement on the presence of low skeletal muscle mass and impaired function or performance as pre-requisites to diagnose sarcopenia. However the use of recommended imaging tools for skeletal muscle mass assessment remains limited by accessibility and costs. Moreover while knowledge of sarcopenia has improved in recent years, Australian specific data are lacking. Hence the aims of this thesis were to examine two cohorts of Australian women with the following goals:

Cohort 1: Australian post-menopausal women above age 40 years

- To explore simple clinical anthropometric measures as a screening tool for sarcopenia, assess the relationship between skeletal muscle mass to bone mineral density, and to assess the relationship between fat mass to bone mineral density in this group (Chapter 2)

Cohort 2: Older Australian post-menopausal women above age 60 years consisting of women with hip fracture compared to those awaiting total hip replacement and healthy controls from the community

- To characterise and compare the hormonal profile for between group differences and to examine circulating hormonal biomarkers for sarcopenia and its relationship to muscle mass and strength in these women (Chapter 3)

- To compare an expanded sex steroid profile including adrenal androgens for between group differences and explore their relationship to muscle mass and strength in these women (Chapter 4)

- To compare signalling proteins in muscle regulation, muscle fibre distribution for between group differences and their relationship to muscle mass and strength in these women (Chapter 5)

The study in Chapter 2 found that skeletal muscle and fat mass were positively associated with bone mineral density over a wide range of ages. Analysis of anthropometric measures shows calf circumference were the strongest predictor of low muscle mass. A calf circumference below 35.6 cm was associated with a sensitivity of 88.6% and specificity of 54.5% in predicting low skeletal muscle mass by DEXA. Leg length remained constant while height declines across age in this cohort. When analysed as a denominator in the skeletal muscle index, leg length were as effective as height in detecting differences in low skeletal muscle mass between groups.

The study in Chapter 3 found that a comparison of hormonal markers reveal low levels of serum Insulin like Growth Factor-1 (IGF-1), Insulin Like Growth Factor Binding Protein 3 (IGFBP-3), beta cell function (HOMA-Beta) and serum testosterone in the most sarcopenic group (hip fracture). There was a positive relationship between these variables to muscle mass and strength supporting their role as potential sarcopenia biomarkers.

The study in Chapter 4 showed that when examining the expanded sex steroid profile and adrenal androgens, low levels of 11-ketotestosterone and 11-ketoandrostenedione were detected in the hip fracture group. Several androgens

were associated with larger skeletal muscle fibre size. Low androgen levels were associated with the presence of increased centralised muscle nuclei indicating increased muscle regeneration activity, contrasting with findings found in other studies in men.

When the intracellular signalling pathways were examined in Chapter 5, the transforming growth factor beta (TGF-β) and muscle ring-Finger (MURF-1) pathways were shown to be the main mechanisms of muscle atrophy in this group. High levels of SMAD 3 accompanied by a compensatory response in SMAD 7 in the TGF-β pathway were observed in the hip fracture group. Additionally MURF-1 levels, a key pathway in skeletal muscle atrophy were highest in the hip fracture group and were consistently associated with low muscle mass and grip strength supporting its role in muscle loss in these women. Women with hip fracture were observed to have atrophied Type I and II muscle fibre. This combined with a lower proportion of Type II muscle fibre observed in the hip fracture group further supports the notion of increased sarcopenia risk in these women.

Taken together these findings provide additional insights into potential surrogate measures to muscle mass assessment, hormonal biomarkers, and regulatory mechanisms underlying muscle loss in older women.

Declaration

This thesis is an original work of my research and contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis. This thesis includes one original paper published in a peer reviewed journal. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Eastern Health Clinical School under the supervision of Professor Christopher Gilfillan.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research. In the case of chapter 3 my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status *(published, in press, accepted or returned for revision, submitted)*	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*
3	Sarcopenia in women with hip fracture: A comparison of hormonal biomarkers and their relationship to skeletal muscle mass and function	Published in Osteoporosis and Sarcopenia Journal, Volume 6, Issue 3, September 2020.	70%. Participant recruitment, collecting data, analysis and draft preparation	1. Raphael Hau 3% participant recruitment 2. Alison Taylor 3% participant recruitment 3. Mark Guerra 3% participant recruitment 4. Peter Guerra 3% participant recruitment 5. Peteris Darzins 3% participant recruitment 6. Christopher Gilfillan 15% Data analysis, supervision, input into manuscript

I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

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I hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work.

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List of abbreviations

4EBP1	4E binding protein1
5-α reductase	5-alpha Reductase
11OH4	11-β hydroxy androstenedione
11BHSD2	11-β hydroxysteroid
11 OHT	11-β hydroxytestosterone
11-KA4	11-ketoandrostenedione
11-A4	11-Androstenedione
11-KT	11-Ketotestosterone
11-DHT	11-Dihydrotestosterone
11-OXO	11 Oxygenated steroids
17OH-Pred	17 α-hydroxy pregnenolone
95% CI	95% Confidence Interval
A4	Androstenedione
ActRIIB	Activin Type IIB receptor
ALM	Appendicular lean mass
ALM-BMI	Appendicular lean mass adjusted for body mass index
AGC	Protein kinase A,G,C
AKT	Protein Kinase B
AMP	AMP activated protein kinase
AR	Androgen Receptor
ATP	Adenosine Triphosphate
β-catenin	Beta catenin
BIA	Bioelectrical Impedance Analysis
BMC	Bone Mineral Content
BMD	Bone Mineral Density
BMI	Body Mass Index
BMP-1	Bone Morphogenetic Protein-1
CT	Computed Tomography
CYP11B1	P450 11β Hydroxylase
D2	Type 2 iodothyronine deiodinase
D3	Type 3 iodothyronine deiodinase
DEXA	Dual Energy X-ray Absorptiometry
DHEA	Dehydroepiandrosterone

eIF4G	Eukaryotic translation initiation factor 4G
eIF4E	Eukaryotic translation initiation factor E
ERK	Extracellular signal regulated kinase
EWSGOP	European Working Group on Sarcopenia in Older people
HR	Hazards ratio
ICU	Intensive Care Unit
IGF-1	Insulin Like Growth Factor-1
IGFBP	Insulin Like Growth Factor Binding Protein
IWGS	International Working Group on Sarcopenia
ICD-10	International Classification of Diseases-10
IRS	Insulin Receptor Substrate
FBBP12	12 kDA FK-506 binding protein
FNIH	Foundation of National Institutes of Health
FNIH-SMI	Foundation of National Institutes of Health-Skeletal muscle index adjusted for body mass index, or SMI-FNIH
FOXO	Forkhead Box O family
GEM	Geriatric Evaluation Management
g/L	grams/Litre
GLUT 4	Glucose Transporter 4
GSK3	Glycogen Synthase Kinase 3
HIF1 alpha	Hypoxia Inducible Factor 1 Alpha
HU	Hounsfield Units
MLST8	Mammalian Lethal with Section 13 Protein 8
MRTF-A	Myocardin related transcription factor A
OR	Odds ratio
MAFbx	Muscle Atrophy F Box
miRNA	Micro ribonucleic acid
mmol/L	millimoles/Litre
MRI	Magnetic Resonance Imaging
mRNA	Messenger ribonucleic acid
mTOR	Mechanistic Target of Rapamycin
MURF-1	Muscle Ring Finger Protein -1
NMJ	Neuromuscular junction
P70S6K	Ribosomal Protein p70S6 kinase
PAX-7	Paired box 7

PCOS	Polycystic Ovarian Syndrome
PGC1α	Peroxisome Proliferator Activated Receptor Gamma Co Activator 1 α
pmol/L	picomoles/Litre
RS6	Ribosomal S6
SPPB	Short Physical Performance Battery
RhoA	Ras homolog gene family member A protein
SARM	Selective androgen receptor modulator
SEM	Standard error of mean
SMAD	Homologues of the Drosophilia protein, mothers against decapentaplegic
SMI	Skeletal muscle mass index
SMI-Baumgartner	Skeletal muscle index (Baumgartner)
SMI-FNIH	Skeletal muscle index adjusted for body mass index (FNIH)
SRF	Serum response factor
STARS	Striated Activator of Rho Signalling
T3	Triiodothyronine
T4	Thyroxine
TSH	Thyroid stimulating hormone
TGF-β	Transforming Growth Factor Beta
ULK1	Unc-51 like kinase 1
Wnt	Wingless and Int 1

Chapter 1: Literature review

1. Introduction

The proportion of the population aged over 65 years will increase in number in the coming years due to better access to healthcare, improved survival and high birth rates in the post-war period. This expansion is expected to occur globally including Australia and New Zealand [1, 2]. By 2050 those age above 60 and 80 years old will increase by 80% and 200% respectively [3]. Considering this trend, it is therefore crucial to address the common conditions affecting this group to enable provision of better services to these individuals.

''Sarcopenia'' is an age-related decline in skeletal muscle mass and function. A loss in skeletal muscle mass begins from the age of 30 at a rate of 0.3-0.8% annually [4]. By age 50 and 70 years, this decline will progressively accelerate to 1% [5] and 1.5% [6]. Initially described by Rosenberg in 1989 [7], multiple studies have consistently shown the association between sarcopenia with adverse outcomes. These include increased falls and fracture risk [8, 9], impaired quality of life [10], poor surgical outcomes [11, 12], prolonged hospital stay [13, 14] and increased mortality in hospital inpatients [15, 16] and those in the community [17]. Furthermore sarcopenia serves as an entry point into the physical cycle of frailty, a separate but overlapping syndrome associated with limited reserve, increased vulnerability and mortality [18].

Efforts into identifying sarcopenia as a significant clinical entity became evident in 2016 when it was listed in the International Classification of Diseases (ICD-10) [19] reinforcing the importance of recognizing and preventing sarcopenia in clinical care. Despite an exponential increase in sarcopenia research in the last few years, insight into this complex condition remains elusive. One reason for this lies in the intricate balance between different pathways involved in skeletal muscle regulation and its interactions with external factors. Moreover sarcopenia definition and cut points in

the literature vary dependant on the different criteria set by expert groups and continues to be debated.

An added challenge to sarcopenia management is the lack of available treatment options. Pharmacological therapies using myostatin inhibitors [20-22] and selective androgen receptor modulators (SARMS) [23] are largely experimental, while hormonal therapies using testosterone and growth hormone are limited by adverse effects. Non-pharmacological therapies such as protein enriched dietary supplementation and resistance exercise have been proven to be beneficial [24-28] and remains the mainstay of sarcopenia management until more promising pharmacological options are established.

In this chapter, a table summarizing the different cut points for sarcopenia definition according to different expert groups are provided. As most of the criteria require the presence of low skeletal muscle mass and function, subsequent sections will focus on the different methods used for assessment of skeletal muscle mass, strength and performance, incorporating the specific cut points used in research studies overseas and the Australian population. The prevalence of sarcopenia is then discussed and concluded with a report on the hormones and protein signalling pathways involved in skeletal muscle regulation.

1.1 Sarcopenia cut points proposed by expert groups

Table 1.1a provides a summary of the specific cut off values suggested by the different expert groups. Commonly used sarcopenia criterion in the literature are the Foundation of the National Institutes of Health (FNIH) and the European Working Group on Sarcopenia in Older People (EWGSOP) criteria. Adjusted values specific to the Asian population are also available from the Asian working group for sarcopenia.

A common criteria in early sarcopenia definitions are the presence of low skeletal muscle mass and strength or function. Over the years as emerging evidence shows an association between reduced grip strength and gait speed with increased disability and mortality [29-32] , these findings have led to a focus on low muscle function and gait speed as a pre-requisite for sarcopenia in later definitions.

While the EWGSOP, International Working Group on Sarcopenia (IWGS) and the Asian Working Group on Sarcopenia guidelines are based on expert consensus, the FNIH criteria were developed from analysis of several large scale pooled studies. A description of the process leading to the development of FNIH criteria are provided in further detail in the discussion of dual energy x-ray absorptiometry (DEXA) as a tool for skeletal muscle mass assessment (Section 1.2.3.1).

Table 1.1a: Summary of sarcopenia definition according to different working groups

Source	**Sarcopenia definition**	**Sarcopenia cut points**
European Working Group on Sarcopenia in Older People (EWSGOP) 2010: Consensus on definition and diagnosis [33]	Low muscle mass and muscle function (Strength or performance)	Muscle mass DEXA <7.26 kg/m^2 in men <5.5 kg/m^2 in women BIA (Bioelectrical impedance analysis) <8.87 kg/m^2 in men <6.42 kg/m^2 in women Muscle strength Men < 30kg Women < 20kg Physical performance Short physical performance battery ≤ 8 Gait speed ‹ 0.8 m/s

Updated EWGSOP Guidelines 2019 [34] Updated EWGSOP Guidelines 2019 continued [34]	Criteria 1: Low muscle strength Criteria 2: Low muscle mass or quality Criteria 3: Low physical performance Probable Sarcopenia: Criteria 1 Confirmed Sarcopenia: Criteria 1 and 2 Severe Sarcopenia: Criteria 1,2 and 3	<u>Low muscle strength</u> Grip strength < 27kg men <16kg women <u>Low muscle mass</u> Appendicular skeletal muscle mass <20kg men <15kg women Appendicular skeletal muscle mass/height ^{2} <7kg/m ^{2} men <5.5kg/m ^{2} women <u>Low physical performance:</u> Gait speed <0.8m/s Short physical performance battery < 8 point score Timed up and Go Test > 20 secs 400m walk test Non completion or > 6 min
Sarcopenia: International working Group on Sarcopenia (IWGS) [35] 2011	Sarcopenia is defined as the age associated loss of skeletal muscle and function	Muscle mass Appendicular fat free mass relative to height^2 ≤ 7.23kg/m^2 in men ≤ 5.67 kg/m^2 in women Gait speed < 1m/s

Sarcopenia in Asia: Consensus Report of the Asian Working Group for Sarcopenia 2014 [36]	Sarcopenia is characterised by age related decline of skeletal muscle plus low muscle strength and/or physical performance	Muscle mass DEXA <7.00 kg/m^2 in men <5.40 kg/m^2 in women BIA <7.00 kg/m^2 in men <5.7 kg/m^2 in women Muscle strength (Grip strength) Men < 26 kg Women < 18 kg Physical performance Gait speed ≤ 0.8m/s
The FNIH Sarcopenia Project: Rationale, Study Description, Conference Recommendations and Final Estimates 2014 [37]	Low muscle mass	Grip Strength Men < 26 kg Women < 16kg Muscle mass Appendicular lean body mass, adjusted for body mass index (BMI) Men < 0.789 Women < 0.512 Appendicular lean mass Men <19.75 kg Women < 15.02 kg Gait speed ≤ 0.8m/s

1.2 Sarcopenia assessment

Sarcopenia is broadly defined by the presence of reduced skeletal muscle mass and muscle strength or performance [33-37]. There are various methods in which these criteria are assessed. Quantification of skeletal muscle mass by magnetic resonance imaging (MRI) and computed tomography (CT) scan are considered gold standard tools for muscle imaging while other alternatives are dual energy X-ray absorptiometry (DEXA), bioelectrical impedance analysis (BIA) and ultrasound.

Reduced muscle strength or performance are essential criteria for the diagnosis of sarcopenia. These measures may be assessed by handgrip strength, gait speed and the Short Performance Physical Battery (SPPB). Over the years, a weak or non-linear relationship were observed between skeletal muscle mass and strength, suggesting a loss in skeletal muscle mass does not equate to loss in muscle function [38-41]. Additionally low muscle strength when compared to low muscle mass, had a stronger relationship with disability and mortality [42, 43]. For these reasons, there is a shift towards the detection of low muscle strength or function as a precursor for investigation of sarcopenia in clinical practice [34, 37].

1.2.1 Skeletal muscle imaging

1.2.1.1 Magnetic resonance imaging (MRI)

MRI images are acquired using data analysed from the interactions between the magnetic field, radiofrequency waves (adjusted by the machine) and hydrogen protons (present in water and fat) which resonate at different frequencies in different body sites. These protons are stimulated when radiofrequency waves are applied [44]. When radiofrequency waves are switched off, signals emitted by the protons returning to their low energy state (in the relaxation phase) are detected and used to create specific MRI images [45, 46].

Skeletal muscle analysis using MRI enables visualization of the muscle in a 3-dimensional manner and assessment of skeletal muscle area. Moreover, the identification of intermuscular and intramuscular lipid [47-49], fibrous tissue and muscle oedema [50] provides additional information on muscle quality. In validation studies, muscle area by MRI highly correlate with corresponding cadaver values ($r=0.99$, $p<0.001$) [51]. Standardized imaging sites for MRI have not been established resulting in the use of different body areas for sarcopenia assessment. A commonly used site for sarcopenia imaging is the truncal and thigh [52, 53] region, followed by calf and paraspinal area.

Low muscle area in the femoral region [54] and increased intramuscular fat infiltration [55] by MRI are associated with impaired physical function in older adults. In those with metastatic colorectal cancer, low erector spinae muscle area is associated with increased mortality [56]. Although MRI provides detailed and precise imaging of the skeletal muscle with no radiation involved, its use is limited by costs, lack of portability and the need for skilled operators to perform and interpret these scans.

1.2.1.2 Computed tomography (CT) scan

CT is another avenue for skeletal muscle analysis. Skeletal muscle area by CT correlate well with values obtained from cadavers ($r=0.99$, $p<0.001$) [51]. Information gathered from CT imaging include muscle cross sectional area and muscle attenuation in Hounsfield units (HU). Due to the distinct attenuation characteristics between muscle and fat, CT can differentiate water (0 HU) from low density fat infiltrated muscle (0 to +29 HU), and normal density muscle (+30 to +100 HU) [57], providing data on muscle quality.

Imaging sites by CT for sarcopenia assessment have not been standardized [58]. As the lower limb muscles contribute to approximately 55-58% of total muscle in the

body [59] the mid-thigh region is a common site used for sarcopenia imaging. In a study to determine which region is a better marker of whole body skeletal muscle, Lee [60] compared muscle area at the 4th and 5th lumbar vertebra to mid-thigh. Using whole body MRI as a comparative measure, CT imaging over both sites correlate strongly with MRI. The mid-thigh region (r^2=0.77-0.90) was a better marker of whole body skeletal muscle than lumbar region (r^2=0.58-0.77).

Many studies have since analysed skeletal muscle over the thoraco-lumbar area [61-63] and psoas muscle [64, 65] to identify individuals with low muscle mass. Critically ill intensive care unit (ICU) patients with reduced muscle at the L3 level have poor outcomes [66], while increased mid-thigh intramuscular fat infiltration are associated with poor physical performance and increased hip fracture risk [67, 68].

Due to the lack of harmonization on specific body sites for sarcopenia imaging, there is currently no consensus on CT cut off values to define sarcopenia. Two studies were performed on kidney donors in the Netherlands [62] and America [63] to derive normative data for CT. Imaging over the thoraco-lumbar region were used, selecting the 5th percentile [62] or 2 standard deviation below the mean of the young healthy population [63] as thresholds for low skeletal muscle mass. Given the retrospective nature of these studies, it remains uncertain if individuals below these thresholds have a risk of future adverse outcomes. A longitudinal study in this regard will provide further information and remains one of the key priorities in sarcopenia research [34].

In specific populations with acute illness, CT is commonly used for skeletal muscle mass assessment [69-71]. Despite a difference in methods used, consistent findings are poor outcomes and increased mortality in those with low muscle mass. Hence in settings where access to MRI is limited, CT is a viable option. The radiation exposure however remains a limiting factor in its use [47] and may be better suited to older

individuals where the potential development of radiation induced malignancy is no longer a concern.

1.2.1.3 Dual Energy Absorptiometry (DEXA)

The utility of DEXA as an alternative to CT and MRI for sarcopenia imaging is gaining interest due to its low running cost and minimal radiation exposure. As a comparison, radiation exposure from a DEXA is estimated at 0.001 mili-Sieverts, significantly lower from that obtained from a chest X-ray [72] and natural background radiation in Australia (1.5 mili-Sieverts, equivalent to 75 chest x-rays) [73]. DEXA has a relatively high precision and correlates well with CT and MRI [58].

DEXA provides an estimate of whole body composition. This allows for a standardized calculation of a skeletal muscle mass index (SMI) adjusted for height, whereby the index is a total of lean appendicular muscle mass (in kilograms) divided by height ^{2} (in metres). The origin of the SMI stems from a cross-sectional study by Baumgartner [74]. A mean of 2 standard deviations below the young healthy adult were selected as criteria for low skeletal muscle mass and was subsequently found to correlate with increased physical disability [75, 76].

This index has since been widely used in sarcopenia research enabling harmonization and comparison of results across different studies. Currently the SMI forms as part of recommended sarcopenia diagnostic criteria by several expert groups [33-36]. Where possible, use of ethnic specific cut-off values is recommended. A commonly used cut point for sarcopenia (defined by presence of low muscle mass and strength) reported in the literature are a SMI < 5.5 kg/m^2 for women, and 7.26 kg/m^2 in men, combined with a grip strength < 30 kg in men or < 20 kg in women using the European Working Group for Sarcopenia in Older People (EWGSOP) definition [33]. Since 2019, the EWGSOP sarcopenia definition has been updated to a SMI <5.5

mg/m^2 and grip strength < 16 kg in women, SMI <7kg/m^2 and grip strength < 27 kg in men [34].

Sarcopenia definition from the Foundation for the National Institute of Health (FNIH) is another popular criteria used in the literature. In 2014, Studenski and colleagues from the FNIH working group [37] reported on their proposed cut points for low skeletal muscle mass in a series of 5 papers [37, 77-80]. Pooled data from 26,625 participants in several large-scale studies were analysed using classification and regression tree analysis [81, 82] to determine clinically relevant cut points. Gait speed < 0.8 metre/second [83] and grip strength < 26 kg in men and < 16 kg in women [77] were selected as primary outcomes because of their association with increased disability and overall reduced survival [37, 83].

By using the above grip strength thresholds to discriminate between 'weak' or 'normal' strength, two cut points for 'low' and 'normal' skeletal muscle mass by DEXA were established [78]:

a) Appendicular lean mass (ALM) <19.75 kg in men, <15.02 kg in women

b) Appendicular lean mass adjusted for BMI (ALM-BMI) < 0.789 m^2 in men, and <0.512 m^2 in women.

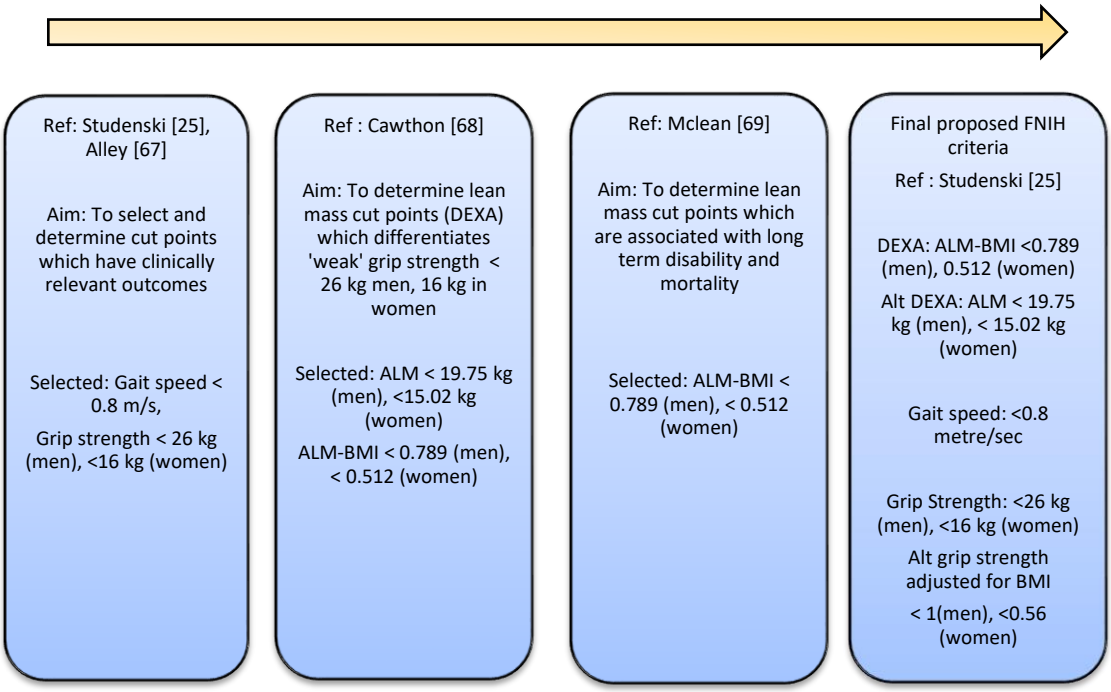
In those with low ALM the odds of being categorised as 'weak' were for men, odds ratio (OR) 6.9, (95%CI 5.4-8.9), and for women, OR 3.6 (95%CI 2.4-4.3), and using the ALM-BMI cut points for men, OR 4.3 (95% CI 3.4-5.5), and for women, OR 2.2 (95% CI 1.8-2.8).

Further analysis by the same group [79] examined the association between these cut points to longitudinal outcomes (impaired mobility and mortality). Only ALM-BMI was associated with an increased risk of future mobility impairment in both genders: men, OR 1.58 (95% CI 1.12-2.22) and women, OR 1.81 (95% CI 1.14-2.87). Figure

1.2.1.3a is a simplified diagram outlining the process of FNIH analysis resulting in the final recommended FNIH criteria. Although not significantly associated with long term adverse outcomes, ALM defined cut points remains as an alternative criteria for low skeletal muscle mass diagnosis.

Figure 1.2.1.3a: Diagram outlining the development of final FNIH criteria

Ref: reference, Alt: Alternative



Use of DEXA for muscle analysis in the Australian population:

There are currently several methods of defining low skeletal muscle mass by DEXA commonly used for research in sarcopenia. These methods along with their relevant cut points to define low skeletal muscle mass are listed:

a) SMI adjusted for height = [Appendicular lean mass (kg)/height² (m^2)]: < 6.7 kg/m^2 in men, < 5.5 kg/m^2 in women (Baumgartner)
b) SMI adjusted for height = [Appendicular lean mass (kg)/height^2 (m^2): < 7kg/m^2 in mean, 5.5 kg/m^2 in women (EWGSOP 2 criteria)

c) SMI adjusted for BMI = [Appendicular lean mass (kg) /BMI (kg/m²)] = ALM-BMI: <0.789 m² in men, <0.512 m² in women (FNIH)
d) ALM < 19.75 kg in men, <15.02 kg in women (FNIH)

For consistency in this thesis, the conventional SMI by Baumgartner will be referred as SMI-Baumgartner, and the SMI (adjusted for BMI) by FNIH (previously referred as ALM-SMI) will be referred to as SMI-FNIH.

To derive normative SMI values for the Australian population, researchers from the Geelong Osteoporosis Group analysed data from a cohort of 2371 individuals recruited from an electoral roll. Reference ranges using the conventional SMI corresponding to 2 and 1 standard deviation below the young (age 20-39 years) mean reference were 6.94 kg/m^2 and 7.87 kg/m^2 in men, and 5.30 kg/m^2 and 6.07 kg/m^2 in women [84]. Values derived from the SMI adjusted for BMI for the Australian population (2 standard deviations below young Australian reference mean) were 0.827 m^2 for men and 0.518 m^2 for women [85]. Further analysis shows good agreement between the SMI-FNIH and the SMI-FNIH (Australian) criteria.

To explore if these values relate to a meaningful outcome, Sim and colleagues compared sarcopenia definitions from four expert groups, assessing its relationship to mortality in older Australian women [86]. Sarcopenia in their study were defined as the presence of low skeletal muscle mass by DEXA and reduced grip strength or function. Cut points from the following were tested:

- SMI-FNIH (SMI adjusted for BMI) <0.512 m² + grip strength < 16kg [37]
- European Working Group for Sarcopenia in Older People 1 (EWGSOP1) (SMI adjusted for height) <5.67 kg/m^2 + grip strength < 20kg and/or timed up and go > 8.3 secs [33]
- Australian adapted version for FNIH cut point / SMI-FNIH (Australian) ALM/BMI <0.517 m^2 + grip strength < 17.1 kg [85]

- Australian adapted version for EWGSOP1 cut point ALM/Height^2 < 5.28kg/m^2 + grip strength < 17.1 kg and/or timed up and go > 8.51 secs [84]

After adjustment for age, low skeletal muscle mass by the EWGSOP1 (adjusted hazards ratio 1.39 95% CI 1.06-1.81, p=0.02) and Australian adapted EWGSOP1 low skeletal muscle mass criteria (adjusted hazards ratio 1.94, 95% CI 1.4-2.69, p<0.01) was strongly associated with mortality risk at the nine year follow up. In contrast neither the FNIH (p=0.11) or Australian adapted FNIH criteria (p=0.62) was found to be associated with mortality in this study [86].

In a separate study, Hirani [87] performed a longitudinal analysis on Australian men using the FNIH criteria of reduced gait speed ≤ 0.8 metre/second, grip strength < 26 kilograms (kg), and appendicular lean mass < 19.75kg [37]. At the five year follow up, those with the presence of reduced grip strength, gait speed and appendicular lean mass were found to have increased risk of disability (OR 4.53, 95%CI 0.9-22.72, p value 0.07), institutionalization (HR 2.27, 95%CI 1.08-4.8, p value 0.03), and mortality (HR 1.69, 95% CI 1.17-2.44, p value 0.01) [87].

In summary, the availability of several cut points using DEXA criteria for low skeletal muscle mass provides the opportunity to compare these values and further evaluate its utility in predicting clinically relevant outcomes in specific populations. While current reports suggests the EWGSOP1 criteria [33] has a stronger association with mortality in Australian women, there is evidence that low appendicular lean mass by the FNIH criteria in Australian men are associated with adverse outcomes [87]. Whether or not these criteria are sex specific remains to be determined and would benefit from further study.

Currently the Australian and New Zealand Society for Sarcopenia and Frailty Research (ANZSSFR) guidelines published in 2018 (before the EWGSOP2 was updated

in 2019) recommend using the EWGSOP criteria [88] for consistency. Ongoing future large-scale collaborative research would provide further insights as to which is a better index for use in the Australian population. A Delphi process on sarcopenia diagnosis and management is underway by the ANZSSFR and we await with interest the outcomes of this.

1.2.1.4 Bioelectrical impedance analysis (BIA)

BIA serves as another option for sarcopenia imaging. A variety of BIA tools are available from different manufacturers. The portability of some BIA machines offers the convenience of bringing the machine to the patient and may address the issues of accessibility faced with MRI, CT or DEXA. Moreover the absence of radiation exposure is an attractive feature.

BIA is based on the concept that the body is a cylinder with measured resistance being proportional to length, and inversely proportional to cross sectional area [89, 90]. A safe low dose electrical current is generated by the BIA machine, channelled through the body by electrodes placed on upper and lower limbs. On a standing BIA machine this is performed with the subject holding metal electrodes. An estimation of fat and lean mass is calculated using different regression equation algorithm programmed into the machine according to manufacturer's guidelines. An estimate of skeletal muscle mass and fat mass is determined by the resistance to the electrical current recorded by the machine whereby fat confers a higher electrical resistance.

Although portable, the accuracy of BIA is limited by altered hydration states particularly in patients on dialysis and diuretics. Furthermore extremes of body mass index will render the results inaccurate [90]. Due to the presence of different regression equations for skeletal muscle mass estimation, there is a broad range of results reported in the literature. Current recommendation suggest that the equation used should be applied to the population in which it was originally assessed [90].

Research into skeletal muscle mass estimation has resulted in the reporting of multiple BIA equations. Sergi [91] and Gonzalez [92] provides a detailed summary of these equations and selected specific cut off values to determine low skeletal muscle mass developed from several groups. Table 1.2.1.4a lists studies which include the older population, their validation tools and regression equations.

Table 1.2.1.4a: Studies in the older population that looked at BIA regression equations

Source	**Regression equation**	**Validation tool**	**Population studied**
Janssen skeletal muscle mass Janssen, 2000 [93]	Skeletal muscle mass (kg)= [height(cm2)/R X 0.401) + gender X3.825 +(ageX-0.071) + 5.102 R=resistance, gender men =1 women=0, age years	MRI	n=388 Age range 18-86 years Caucasian, Hispanic, African American, Asian 2 laboratories in UK/Canada
Kyle Appendicular skeletal muscle mass Kyle, 2003 [94]	Appendicular skeletal muscle mass (kg) =-4.211 + [0.267 X height $(cm)^2$/resistance + (0.095 X weight) + (1.909 X sex) + (0.012 X age) + (0.058 X reactance) height in cm, men=1, women=0	DEXA	n=770 444 healthy adults (age 20-94) compared against 326 organ transplant patients (age 18-70) Switzerland
Tengvall skeletal muscle mass Tengvall, 2009 [95]	Skeletal muscle mass = -24.021 + (0.33 X height) + (-0.031XR) + (0.083Xreactance) + (0.58X sex) + (0.046 X weight) R=resistance, react=reactance weight =kg women=1, men=0	DEXA	n=672 Healthy older adults age 75 Sweden

The Janssen study was performed on a group of multiethnic participants aged between 18-86 years old in Canada and United States of America. A prediction equation developed by multiple regression modelling was validated against skeletal muscle mass measured on MRI [93]. Reported r^2 value was 0.86, accounting for 86% of the variance with a prediction error of approximately 2.7 kg. Further analysis shows that the regression was valid in the Caucasian, Hispanic and African-Americans, but underestimates skeletal muscle mass in the Asian population [93].

This equation was further evaluated in a group of 4449 older participants (age ≥ 60) in a separate study. Skeletal muscle mass cut points adjusted for height of ≤ 5.75 kg/m^2 and 6.75 kg/m^2 for women, and ≤ 8.5 kg/m^2 and 10.75 kg/m^2 in men were found to increase the odds of physical disability by 3.31 and 1.41 in women, and 4.71 and 3.65 in men [75].

In a study by Kyle in Switzerland [94], 770 participants consisting of 444 healthy participants age between 20-94 years old and 326 organ transplant patients were examined. A regression equation was first developed in healthy participants and was further validated in organ transplant patients. The equation derived to predict appendicular skeletal muscle mass were shown to strongly correlate with DEXA. Reported r^2 values were 0.95 and 0.91 with a standard error of estimate (SEE) of 1.1 and 1.5 kg in healthy participants and patients, supporting the use of this equation in both groups.

The Tengvall [95] study is a Swedish study looking at a cohort of individuals aged 75 years old. Data from 98 individuals who had both DEXA and BIA were compared against 574 other individuals who had BIA. The BIA regression equation developed for estimation of skeletal muscle mass had a strong correlation with DEXA (r^2 value

0.91) [95] and have been used in studies on hip fracture patients [96] and older hospital inpatients [97].

To determine if these equations are applicable to the Australian population, Bosaeus compared the Kyle, Janssen and Tengvall prediction equations for skeletal muscle mass [97] in hospital inpatients. When compared against DEXA, regression equations by Kyle and Janssen overestimate skeletal muscle mass by 1.45-2.05 kg, while the Tengvall equation were found to underestimate skeletal muscle mass by 1.79 kg. Although the Kyle equation appears to have the lowest observed discrepancy in measured skeletal muscle mass between BIA and DEXA, repeat studies comparing these different equations on an Australian population would be beneficial to determine if this is a consistent finding. This will provide further insights into its applicability to the Australian population.

1.2.1.5 Ultrasound

Ultrasound can be used to measure muscle thickness and echogenicity [50]. The lack of standardized imaging site and specific cut off value to define low skeletal muscle mass by ultrasound remains another challenge. Predominant areas used for imaging include the vastus lateralis, rectus femoris and the anterior abdominal muscles [98]. Compared to other imaging tools discussed earlier, its utility in skeletal muscle research is still rudimentary. Despite this, the use of ultrasound as a potential tool for skeletal muscle mass assessment remains an option [33, 34] due to the absence of radiation and continues to be explored [99-102].

1.2.2 Sarcopenia assessment: muscle strength and performance measures

Reduced skeletal muscle function or strength is an essential criterion for the diagnosis of sarcopenia. The latest guidelines [34, 37] emphasized screening for muscle strength or gait speed as a trigger for investigation of sarcopenia.

1.2.2.1 Grip strength

Reduced grip strength forms as part of the recommended testing criteria for sarcopenia because of its association with impaired nutritional status [103], increased disability and mortality [29, 30, 104, 105]. It is a simple assessment conducted using a handheld dynamometer with the study participant ideally seated in a chair with back support and arms at 90° angle. The participant is instructed to squeeze the handle of the dynamometer tightly until the needle stops rising and grip strength is read closest to the 1 kilogram [106]. Results obtained from grip strength assessment can vary depending on the type of dynamometer used, holding position, number of attempts [106] and degrees of arthritis affecting the hands [107].

Although grip strength is recommended by current guidelines for sarcopenia assessment, the choice of dynamometer or preferred method have not been specified. The Jamar handheld dynamometer is generally accepted as the preferred tool given its good test-retest reliability [108] and most data in the literature were gathered using this device [106].

Current specific cut points to define low grip strength are < 26 kg in men and < 16 kg in women based on the EWGSOP2 and FNIH guidelines [34, 37]. Selection of these values from the EWGSOP2 group was based on a study of twelve population studies in Great Britain by Dodds [109]. Using normative data for grip strength collected across the life span, grip strength values corresponding to the 2.5 standard deviation below the mid to late adult life were ≤ 27 kg in men, and < 16 kg in women.

In contrast, in the FNIH study series by Alley [77], selection of their cut points were based on isolating a grip strength that was able to detect mobility impairment, defined by a gait speed of < 0.8 metre/sec. A grip strength < 26 kg in men, <16 kg women was associated with a high likelihood of mobility impairment [men: OR 7.62 (95%CI 6.13-9.49), women OR 4.42 (95%CI 3.94-4.97)]. However, it is important to

note that a grip strength < 32 kg in men and < 20 kg in women still had an OR for mobility impairment of 2.44 for women and 3.63 for men in this study. In favour of selecting cut points which can identify a significant degree of weakness with stronger confidence, the authors adopted a conservative approach by selecting lower cut points [77].

Currently, Australian specific grip strength cut off values for sarcopenia definition are not available. A study performed in Adelaide documenting normative grip strength data for the population reported a mean grip strength of 20-23 kg in women, and 32-40 kg in men in those age above 60 years. However, values corresponding to the 2.5 standard deviation below the mean were not reported [110].

At present consensus from the ANZSSFR in 2018 [88] suggest adopting the EWGSOP1 criteria (since updated to EWGSOP2 in 2019). Grip strength for sarcopenia definition are as listed:

- EWGSOP 1 :< 30 kg in men, < 20kg in women [33]
- EWGSOP 2: <27 kg in men, < 16 kg in women [34]

Current evidence from studies on Australian participants suggests that EWGSOP1 (grip strength < 16kg) are associated with increased mortality in women [86] and FNIH criteria (grip strength < 26kg) are associated with increased disability and mortality in men [87]. Whether or not cut points from the EWGSOP 2 criteria are better suited to the Australian population requires further evaluation, given recent work by Villani [111] reporting significant discordance between the new and old EWGSOP definitions.

1.2.2.2 Functional assessment: Gait Speed

Reduced walking speed is associated with increased hospital readmission rates [112], disability and mortality [31, 83]. Gait speed is measured by the time taken for an individual to walk over a 4 metre distance at their usual speed. Current guidelines proposed using a cut off level of < 0.8 metre/second to define low muscle performance [34, 37].

1.2.2.3 Functional assessment: Short Physical Performance Battery (SPPB)

As an alternative to grip strength and gait speed, the use of Short Physical Performance Battery has also been recommended by the EWGSOP2 for assessment of muscle function. This tool has been developed by the National Institutes of Aging [113] and includes a series of tests for balance, gait speed and chair stand test. A score of < 8 on the tool is used to define sarcopenia, and reduced SPPB scores have been shown to be a predictor of all-cause mortality [114]. Other functional tests proposed in the updated EWGSOP 2 guideline include the use of Timed up and Go, Chair Stand Test and the 400-metre walk test.

1.2.3 SARC-F questionnaire as a screening tool

The SARC-F questionnaire [115] (Table 1.2.3a) was developed as a simple screening tool and has been validated in several studies [116-118]. Sarcopenia was defined as SARC-F score ≥ 4. Scores above this have been shown to be associated with increased disability, hospitalisation and mortality [119]. Reported sensitivities for this test were generally low (5-34%) with higher specificities (80-97.5%). In the assessment of the Australian population using this tool, 51% of patients from a high risk foot clinic with diabetic foot ulcer were considered sarcopenic with reported reduced quality of life [120]

Table 1.2.3a: SARC-F Questionnaire [115]

Component	Question	Scoring
Strength	How much difficulty do you have in lifting and carrying 10 pounds	None=0 Some=1 A lot or unable =2
Assistance in walking	How much difficulty do you have walking across a room?	None=1 Some=1 A lot, use aids or unable =2
Rise from a chair	How much difficulty do you have transferring from a chair or bed	None=1 Some=1 A lot or unable without help=2
Climb Stairs	How much difficulty do you have climbing a flight of 10 stairs?	None=0 Some=1 A lot or unable =2
Falls	How many times have you fallen in the past year?	None=0 1-3 falls =1 4 or more falls =2

1.3 Sarcopenia prevalence

Sarcopenia prevalence overseas:

The prevalence of sarcopenia varies dependant on the criteria used and population assessed. In the Baumgartner study in the community thresholds for sarcopenia were defined using a SMI 2 standard deviation below the young age group mean. In

this study, the prevalence of sarcopenia was 13-24% in those younger than 70 years, 18-36% in the 71-80 age group, and 43-60% in those older than 80 years [74].

In the European community, sarcopenia prevalence range between 20 to 29% [26, 121], 10-42% in hospital inpatients [26, 122] and 17-73% in residents living in aged care facilities [123]. In most of these studies, sarcopenia was defined using the EWGSOP criteria [33, 34].

Sarcopenia prevalence in Australia:

In the Australian community approximately 2.5-45% men and 3.2-37% women [86, 124-127] were considered sarcopenic. In contrast the prevalence of sarcopenia in residential age care residents was higher ranging between 25-40% [127-129]. The large variation observed in these numbers is related to the different methods used in sarcopenia assessment. While most of the studies use EWGSOP1 criteria, several others have also defined sarcopenia using the FNIH criteria [37, 86, 124], appendicular lean mass adjusted to fat mass [126], and skeletal muscle index percentage [127].

There are currently no data on sarcopenia prevalence in Australian acute hospital inpatients. However it is likely that sarcopenia would be more prevalent in this group compared to the general population. With regard to the patients on the rehabilitation wards and geriatric evaluation management (GEM) unit, two studies were performed. Perez-Zepeda in Adelaide examined GEM inpatients using the EWGSOP1 criteria. Approximately 40.1% of their patients were sarcopenic with a two-fold increase in mortality at the 12 months follow up [16]. In Melbourne, another study has just completed recruitment in the rehabilitation wards and we await further reporting [130].

In Australians with specific medical conditions, there were a significant proportion of sarcopenic individuals in each group and rates were considerably higher than the general population. Reported sarcopenia prevalence was 71% in the hip fracture population [131], 12-21% in those with Crohn's Disease [132], 38-70% in individuals with liver cirrhosis [133], and 51% in those with diabetic foot ulcers [120].

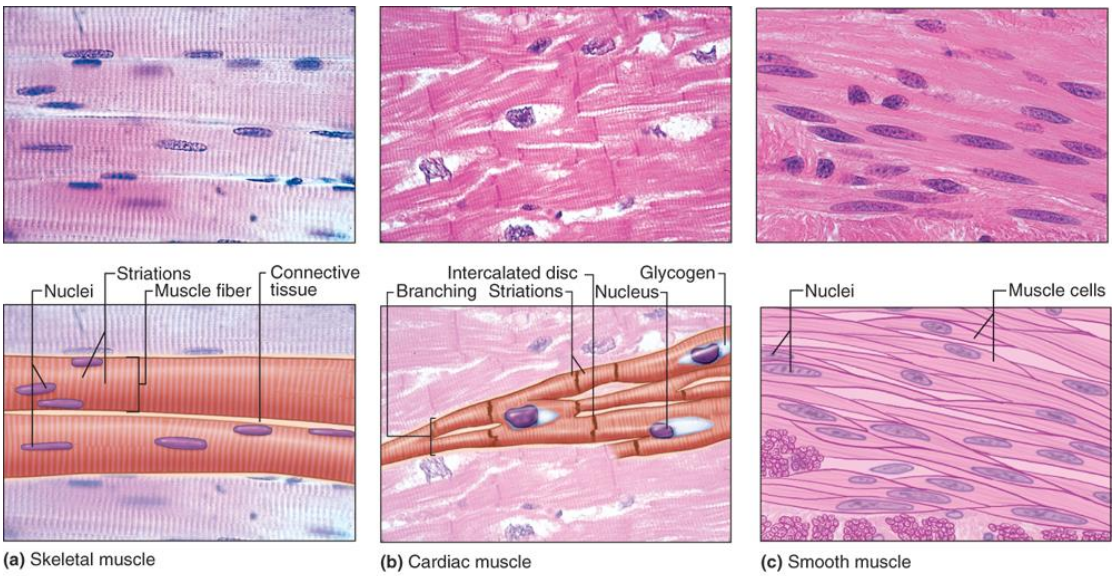
Data regarding sarcopenia in Indigenous Australians are scarce. Although low grip strength [134] and musculoskeletal diseases [135] are common in this group, the prevalence of sarcopenia is not known. This is due to the absence of normative data [135] thereby limiting comparison. This is an important area for future research.

Overall current knowledge suggest the prevalence of sarcopenia increase with older age, is more common in those with chronic illness and individuals in residential aged care facilities. Individuals with hip fracture may be at especially high risk given the high incidence reported [131, 136-138]. Understanding the basic mechanisms and cellular changes underlying skeletal muscle regulation is therefore an important agenda in skeletal muscle research. Improved insights will ultimately provide a guide towards exploration of potential treatment options for sarcopenia.

1.4 Cellular changes in ageing muscle

There are 3 types of muscle tissue in the body: skeletal, cardiac and smooth muscle. Characteristic features of the skeletal muscle are the presence of multinucleated cells with long bundles of myofibrils organised in a regular pattern giving the light microscopic finding of striations. Skeletal muscle contractions are voluntary and forceful. In contrast, cardiac muscle is identified by the presence of intercalated discs with involuntary, rhythmic contractions while smooth muscle are characterised by the lack of striations and slow involuntary contractions [139]. Figure 1.4a is an image showing these features. Discussions in the subsequent sections will focus predominantly on skeletal muscle and the changes observed with ageing.

Figure 1.4a: Light microscopy histology features of skeletal, cardiac and smooth muscle, adapted from Mescher 2018 [139].



1.4.1 Alterations in Neuromuscular junction (NMJ)

The motor unit was initially described by Liddell and Sherrington in 1925. It consists of a single alpha motor neuron which branches into axons to innervate the skeletal muscle fibres [140]. For skeletal muscle contraction to occur, electrical impulses from the anterior horn cell is propagated along the nerve axons which then terminate at the motor end plate also known as the neuromuscular junction (NMJ). This results in increased permeability of the motor neuron membrane to calcium, leading to an influx of calcium with the subsequent release of acetylcholine into the synaptic cleft. Acetylcholine then binds to its receptors on the sarcolemma of the skeletal muscle, resulting in Na+/K+ exchange, depolarization of the sarcolemma and muscle contraction through excitation contraction coupling [140, 141].

Increase age is associated with a progressive decline in the motor unit and alterations in the NMJ milieu supplying the skeletal muscle fibres [140, 142]. While this is a consistent observation reported in the literature, the specific triggers and mechanisms underlying this decline remains unclear [140, 143]. Proposed

mechanisms include impaired signalling from the central nervous system, alterations in trophic signalling from the diseased muscle and local degeneration [144]. At the level of the NMJ, inhibition of neural agrin (a signalling protein with an important role in the maintenance of NMJ) results in disassembly of the NMJ within days [145], and fragmentation of the NMJ [143] have also been reported as contributing factors.

During adult life it is believed that the skeletal muscle undergoes remodelling with repeated cycles of denervation and reinnervation [140, 142, 145]. The exact cause for this transient denervation remains uncertain. Apart from alterations in NMJ signalling, other hypothesis for this observation include a sudden change resulting in axonal degeneration or a traumatic injury leading to myofibre necrosis and altered NMJ morphology [145]. During the process of transient denervation, the skeletal muscle fibre is disconnected temporarily from its motor axon followed by reinnervation either by the original supplying axon or collateral sprouting of new axons from the adjacent surviving motor neuron [145]. Denervation have also been reported to increase mitochondrial reactive oxygen species production leading to the activation of autophagy pathway via Forkhead Box O family (FOXO) [145], discussed in the later sections on muscle regulatory pathways.

In an older skeletal muscle, the process of repeated denervation and reinnervation is thought to be exaggerated [142] resulting in clustering of a single muscle fibre type close to one another and the presence of flattened, smaller muscle fibres with a more angular shape [145]. This is in contrast to findings in a young skeletal muscle, whereby there is a mixture of different muscle fibre types in a mosaic distribution with contiguous bands giving the typical ‘pretzel’ like appearance [145, 146]. (Figure 1.4b).

Figure 1.4b Alterations in the ageing muscle resulting in a change in fibre type grouping [145]. Adapted from Hepple, 2016.

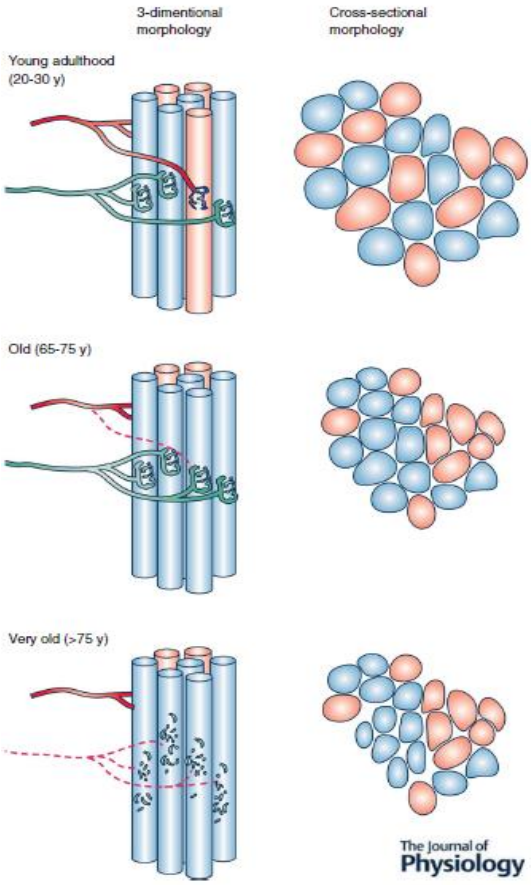


Figure 1.4b shows the cross sectional and 3 dimensional morphology of skeletal muscle fibres from a young, old and very old individual.

Loss of motor neuron is observed from the seventh decade onwards contributing to the deterioration and progressive loss of motor unit. In the very old muscle (>75 years), this change results in clustering and smaller atrophied muscle fibres observed on cross-sectional morphology when compared to muscle fibres from younger individuals (age 20-30 years).

Colours in the skeletal muscle fibres viewed on cross sectional imaging corresponds to their innervating NMJ.

1.4.2 Reduced satellite cell number with aging

Skeletal muscle satellite cells are skeletal muscle stem cells that play an important role in skeletal muscle regeneration. Satellite cells lie in a dormant state between the sarcolemma and basal lamina [147]. In the presence of stimulatory factors (IGF-1, exercise, interleukin 6), these cells are activated to differentiate into mononucleated myoblasts [148]. Fusion of myoblasts results in concentration of the nuclei in the central compartment, followed by migration of the nuclei along the long axis as the mature myofibres develops [149]. Skeletal muscle is typically identified by its hallmark peripheral nuclei [149] while the presence of non-peripheral, central nuclei suggests regenerating muscle fibres [150].

Aging is associated with a reduction in satellite cell number [147], function and impaired regeneration capacity. Several factors are thought to contribute to this including somatic mutations in the satellite cell genome and alterations in the cellular environments and signalling pathways [151]. In older men, administration of androgens [152] and exercise [153] has been found to increase satellite number in these individuals.

1.4.3 Muscle fibre loss and atrophy with ageing:

Skeletal muscle fibres are categorised into Type I (slow twitch) and Type II (fast twitch) muscle fibres, dependant on their maximal rate of contraction and pathways utilised to generate adenosine triphosphate (ATP), either by oxidative phosphorylation or glycolysis [139].

Type I or slow oxidative type skeletal muscle fibres are characterised by the presence of multiple mitochondria, abundant capillaries and intracellular lipid for oxidative aerobic metabolism. These fibres contain a high myoglobin (iron/oxygen binding protein) content typically giving it a dark or red colour appearance. Type I fibres are recruited first in response to gradual increasing muscle load. Although they have slow contractions and

generate less force than Type II fibres, Type I fibres are able to contract over prolonged periods with less fatiguability [139, 154].

In contrast Type II muscle fibres are fast twitch glycolytic fibres. They are dependent on anaerobic metabolism of glucose from stored glycogen to generate ATP. These fibres typically have smaller mitochondria, capillaries and intracellular lipid with larger glycogen stores. Due to the lack of myoglobin content these muscle fibres appear white. They are recruited late following increasing muscle load and are utilised for intense short burst activities.

Type II muscle fibres fatigue easily due to the accumulation of lactic acid from glycolysis [139, 154]. This group of muscle fibres can be further categorised into three different types based on their myosin heavy chain gene expression, Type IIa, IIx and IIb. Of these three, only Type IIa and IIx fibres are detected in humans [155]. Table 1.4a from Hill, 2019 provides a summary of these features [156]. Figure 1.4c shows the different muscle fibre types identified by anti-myosin antibody.

Table 1.4a Different characteristic features of muscle fibre type adapted from Hill, 2019 [156]

Fibre Type	**Type I**	**Type IIa**	**Type IIx**	**Type IIb**
Contraction time	Slow	Moderately Fast	Fast	Very Fast
Size of motor neuron	Small	Medium	Large	Very Large
Resistance to fatigue	High	Fairly High	Intermediate	Low
Activity used for	Aerobic	Long term Anaerobic	Short term anaerobic	Short term anaerobic
Maximum duration of use	Hours	<30 minutes	< 5 minutes	<1 minute
Power produced	Low	Medium	High	Very High
Mitochondrial Density	High	High	Medium	Low
Capillary Density	High	Intermediate	Low	Low
Oxidative capacity	High	High	Intermediate	High
Glycolytic capacity	Low	High	High	High
Major storage fuel	Triglycerides	Creatine phosphate, glycogen	Creatine phosphate, glycogen	Creatine phosphate, glycogen

Fibre type switching between Type I, IIa and IIx occurs dependent on the denervation-reinnervation activity in addition to the nerve in which it is supplied. In animal models following injury, fast type muscle fibre gene expression was observed in denervated muscle while functional reinnervation of regenerating muscle fibre favours the upregulation of slow type muscle fibre gene expression [157]. Fibre type switching are also influenced by the electrical stimulation provided by the supplying nerve. High frequency electrical stimulation induce the conversion of slow Type I muscle fibre to fast twitch Type IIa, IIx and IIb muscle fibre. Conversely conversion of Type II to Type I muscle fibre occurs with low frequency electrical stimulation [157]. In the process of skeletal muscle regeneration following injury, Type II muscle fibres are first expressed followed by a switch to slow Type I muscle fibre detected 5 days after injury [158].

Figure 1.4c Human skeletal muscle fibre types identified by anti-myosin antibodies, adapted from Schiaffino, 2018. Blue indicates Type I, Green indicates Type IIa, Red indicates Type IIx muscle fibres. In yellow asterisks are hybrid fibres, indicating the presence of both Type I and II muscle fibres [159] .

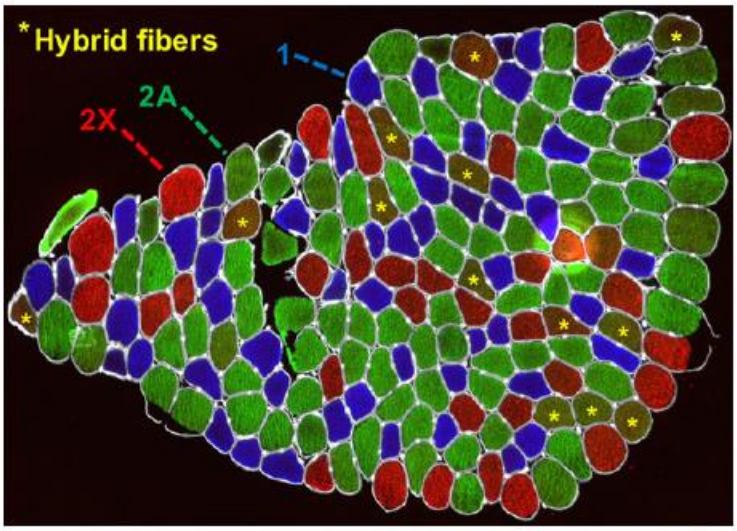
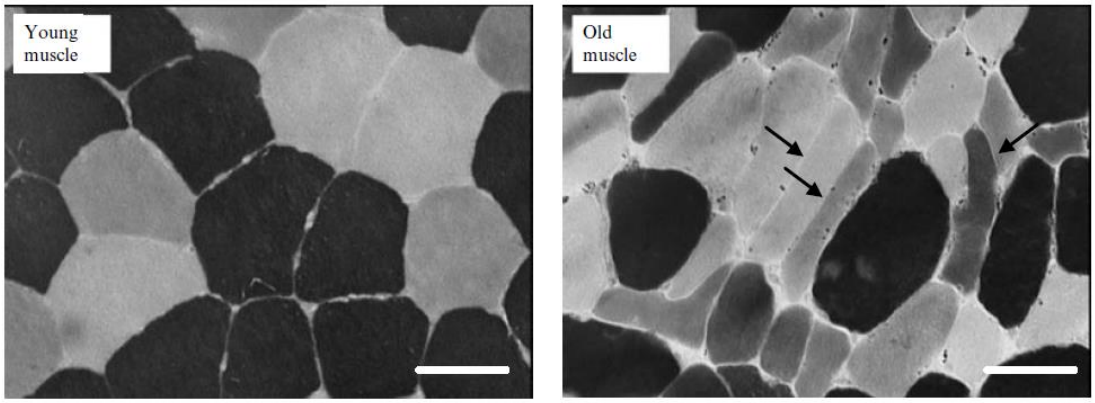


Figure 1.4d Muscle fibre types in a young and old skeletal muscle. Dark fibres: Type I fibres, White fibres: Type IIa, Grey fibres: Type IIx adapted from Andersen 2003 [146]. Muscle fibre from the younger specimen shows angular fibres whilst muscle fibre from the older study participant shows flattening of the muscle fibre with these changes more pronounced in the Type II muscle fibres.



Aging is associated with muscle fibre loss and atrophy [160] (Figure 1.4d). While most studies suggest a preferential loss and atrophy with Type II muscle fibres in the older population [161-163] there are also reports of both Type I and Type II muscle fibre loss in

this group [164, 165]. Given Type II muscle fibre are involved in short burst activities, a loss in Type II muscle fibre number may be associated with increased falls risk [162].

To examine this, studies have been performed in the hip fracture population evaluating muscle fibre type distribution. Scimeca [166] compared muscle biopsies in older patients with osteoporotic hip fracture (mean age 71) to those with osteoarthritis (mean age 73) and younger patients with hip fracture from high impact injury (mean age 40). Compared to younger controls, the older age groups were found to have a higher percentage of atrophied Type I and Type II muscle fibres. There were equal proportions of atrophied Type I and Type II muscle fibres in the osteoarthritic group, contrasting with a higher proportion of atrophied Type II muscle fibre in the osteoporotic group. Staining with Pax 7, a marker of satellite cell presence were increased in the osteoarthritic compared to the osteoporotic group, suggesting impaired muscle regeneration as a mechanism to muscle loss in the osteoporotic group.

In another study by Kramer [162], they compared older women with hip fracture to healthy age matched controls and young healthy controls. Compared to all groups, women in the hip fracture group were observed to have significant Type II muscle fibre atrophy. These women were also observed to have significantly smaller Type I muscle fibres when compared to age matched cohort.

Overall ageing is associated with muscle fibre loss and atrophy in both muscle fibre types. Although most studies suggest a preferential loss and atrophy in Type II muscle fibre, there are also reports of loss and atrophy in Type I muscle fibres. These differences may be explained by the variation in methodology between studies and location of muscle biopsies. Furthermore given androgens have been shown to increase muscle area of both muscle fibre types [152], and muscle fibre type switching is dependent on electrical stimulation

(high vs low frequency), it is likely that the effect of other confounding factors, i.e. hormonal factors and nerve supply have not been accounted for in the analysis.

1.5 Hormonal regulation on the skeletal muscle

Muscle homeostasis is influenced by multiple factors including the presence of different hormones and will be discussed in the following sections.

1.5.1 Testosterone

Testosterone is part of a group of C19 androgenic steroids produced from its precursor cholesterol. In men testosterone is responsible for the development of secondary sex characteristics with anabolic effects on the muscle and bone. Given the differences in sex hormones and their effects in men and women, discussion will first focus on testosterone in men, followed by a discussion of the role of androgens (including testosterone and 11-oxygenated steroids) in women, and our current understanding of the actions of androgens on skeletal muscle at the cellular level.

Testosterone in men:

Testosterone is primarily produced by the testes and peaks during puberty in men. It is required for the development of secondary sexual characteristics, stimulation of sexual function and initiation of spermatogenesis. At its target tissues testosterone is converted into a more potent form of androgen, dihydrotestosterone by 5-α reductase. Its anabolic properties include stimulation of bone growth, increase in bone mineral density and skeletal muscle mass and strength [167]. Testosterone levels decline with aging in men [168] and is thought to contribute to changes in body composition as reflected by an increase in fat mass and reduced lean body mass. This observation is supported by studies in men with androgen deprivation therapy for the treatment of prostate cancer who experience similar changes in body composition [169].

Testosterone supplementation is associated with increase in skeletal muscle mass and muscle strength [170, 171]. In hypogonadal men, testosterone replacement is associated with increased skeletal muscle protein synthesis [172], resulting in increased skeletal muscle mass and strength [173]. Similar changes in body composition were also observed in eugonadal men, with responses being dose dependant [174]. These findings have led to trials using supraphysiological doses of testosterone in older eugonadal men [175]. In one study, weekly doses of testosterone enanthate (25-600 mg) were administered to a group of young and older eugonadal men. Compared to the younger group, older men were equally responsive to the anabolic effects of testosterone, with significant gains in lean mass and leg press strength at higher doses of testosterone. However the older age group also reported increased incidents of adverse events (increased haematocrit, leg oedema, prostate cancer) limiting its use as a potential therapy for sarcopenia.

Another study looked at the use of topical testosterone in older men with low or low normal testosterone levels [176]. Study participants were randomised to either topical testosterone or placebo. Treatment was continued for a period of 3 years and participants were followed up at 6, 18 and 36 months. Although there was an increase in stair climb, chest press and leg press in the treatment group, the differences between group improvements in these parameters were observed in the first 6 months followed by a gradual decline thereafter. While differences between placebo and treatment group remained significant at the end of the 3 year study these gains were modest. In a similar study over a 1 year period, topical testosterone was combined with supervised resistance exercise training in older men. Improvements in body composition were observed but no change in muscle function [177].

At present testosterone supplementation in men is only indicated for those with hypogonadism [178]. Although there are gains in body composition with testosterone supplementation in older men, functional improvements were minimal with inconsistent results. Moreover the need for supraphysiological doses to achieve significant results combined with the risks of adverse effects (increased haematocrit, exacerbation obstructive

sleep apnoea and prostate cancer, bladder outlet obstruction) far outweighs the gains to justify its use for sarcopenia at this stage.

For this reason attention has turned to the selective androgen receptor modulators (SARMS). In 1998, Dalton and colleagues [179] examined a group of ligands to determine their binding to the androgen receptor and activation of receptor mediated transcriptional activation. Their study isolated three ligands which bind to androgen receptors and were able to activate transcriptional activation in a similar manner to dihydrotestosterone (a potent androgen) with selectivity for certain tissues. This finding resulted in studies examining the effect of SARMS on the skeletal muscle.

In a phase 2 trial the use of enobosarm was evaluated for 12 weeks in a group of older individuals (n=120) [23]. This trial included both men and women. At the end of treatment there was a significant but clinically modest increase in lean mass (+1.3kg), decrease in fat mass (-1.3kg), and shorter stair climb speed time (-0.8 seconds). Reported adverse events were a small but significant increase in haematocrit, and a transient rise in alanine amino transferase (ALT) levels resulting in one participant stopping treatment. Importantly in men there were no reports of prostate cancer or virilization in women. Although these results appear promising it is uncertain if the effects are sustained due to the nature of the trial. Therefore a study of longer duration would be helpful in providing further insights.

Testosterone and 11-OXO androgens in women:

In women, testosterone is primarily produced by the ovaries [180] and by peripheral conversion of androgen precursors [androstenedione and dehydroepiandrosterone (DHEA)] produced by the ovaries and adrenal glands [180, 181]. Testosterone production in women peaks during the third and fourth decades of life and subsequently starts to decline thereafter [180, 182].

In recent years the 11-oxygenated (11-OXO) steroids, another group of androgens produced by the adrenal glands are gaining increasing interest. Conventionally thought to have negligible androgenic activity, its clinical relevance particularly in those with androgen excess [congenital adrenal hyperplasia and polycystic ovarian syndrome (PCOS)] is becoming more apparent [183, 184]. Moreover these group of steroids are now considered a group of major bioactive androgens in women [185] and may be as potent as testosterone [183]. Unlike testosterone, levels of the 11-OXO steroids do not decline with age [186]. This feature makes it an attractive hormone for studies in the older population.

In women, testosterone exerts both androgenic and oestrogenic effects (via the aromatisation of testosterone) at different target sites [180]. Observational studies have shown a positive association between testosterone levels with increased lean body mass and fat mass [187]. Those with low free testosterone and DHEA levels were observed to have reduced muscle strength [188]. While it is not known if 11-oxygenated steroids are associated with changes in lean mass, women with PCOS (where 11-oxygenated steroids are elevated) were reported to be stronger and more muscular than their non PCOS counterpart [189, 190]. Given this observation there may be a possible link between the 11-oxygenated steroids and skeletal muscle. This hypothesis is explored in this thesis (Chapter 4).

Systematic reviews examining the effect of testosterone supplementation in post-menopausal women have reported an improvement in those with low libido [191], however no significant effects were reported in terms of body composition [192]. In clinical trials using androgens in post-menopausal women, testosterone was associated with improved bone mineral density in the vertebral and trochanteric area [193], but mixed results were observed with the use of DHEA and muscle performance [194, 195]. A small study using 14 mg of oxandrolone for 2 weeks in older women observed an increase in muscle protein synthetic rate but lean mass or function were not reported [196]. Although there are some benefits in the use of androgens on muscle and bone, the need for supraphysiological doses of testosterone to achieve significant muscle gains seen in trials in men is unlikely to occur

in women given the risks of virilization and ethical issues. At present the use of testosterone in post-menopausal women is limited to those with female sexual dysfunction [197].

Another use of testosterone in women in the clinical setting is in transgender men. In these individuals where testosterone therapy are administered to achieve secondary male sexual characteristics, there are reports of increased lean mass, muscle strength and reduced body fat [198, 199] in body composition studies.

Studies using SARMS as a potential option for sarcopenia treatment also included older women. Those who were treated with SARMS showed modest improvements in muscle mass and stair climb speed [23]. In another study evaluating the use of a different SARM, MK-0773 in frail sarcopenic women for 6 months, no changes were seen in muscle performance despite an increase in lean body mass [200]. Based on these results it is still too early to recommend SARMS for sarcopenia treatment. Studies of longer duration would be helpful to determine if the increase in muscle mass have any meaningful clinical impact in the long term.

Cellular mechanisms of androgens on the muscle:

Androgen receptors (AR) are present in the human skeletal muscle, and treatment with androgens upregulates the expression of AR protein [201]. Although the anabolic properties of androgens on the muscle are known the exact cellular mechanisms are not fully understood. Trials using androgens in men have observed an increase in myonuclear and satellite cell number [202], muscle fibre hypertrophy, and increase in muscle fibre cross sectional area in Type 1 and Type 2 muscle fibres [152, 203].

The AR can act via the classical and non-classical pathways to affect the skeletal muscle [204, 205]. Following binding of androgens to the intracellular AR, there is a conformational change in the AR, dissociation from heat shock proteins and the subsequent translocation of

AR to the nucleus to initiate gene transcription and protein synthesis [167]. This process occurs via the classical (DNA-binding dependant) pathway.

AR can also function in a non-classical (non-DNA-binding dependant) manner [204] through the phosphorylation of 2nd messenger signalling cascades. These include interaction with the mitogen activated protein kinase (MAPK), extracellular regulated kinase (ERK) and protein kinase B (AKT) [204], pathways involved in cell growth and proliferation. AKT is also part of the mechanistic target of rapamycin (mTOR) pathway which will be discussed later in section 1.6.1.

Androgens via the AR have also been reported to encourage differentiation of pluripotent cells towards the myogenic lineage [205], and inhibit myostatin a member of the transforming growth factor-β (TGF-β) superfamily which inhibits skeletal muscle growth. This process is thought to occur via upregulation of the β-catenin-wingless and Int 1 (Wnt) signalling pathway resulting in increased follistatin expression [206]. As follistatin inhibits myostatin, its upregulation results in increased cell growth. Combined, these findings suggest androgens affect skeletal muscle through diverse pathways most of which are interlinked with other signalling pathways.

1.5.2 Insulin Like Growth Factor – 1 (IGF-1)

Growth hormone is produced by the pituitary gland and triggers the secretion of Insulin Like Growth Factor-1 (IGF-1) from the liver. Unbound IGF-1 has a short half-life (few minutes) [207]. In view of this, the majority of serum IGF-1 in the circulation are bound to IGF binding proteins (IGFBPs) and an acid labile subunit thus prolonging its half-life to up to 16 hours [208]. Interaction between IGF-1 and its receptor leads to a conformational change and activation of tyrosine kinase receptor. This initiates a series of downstream signalling cascades [208] resulting in the activation of the mechanistic target of rapamycin (mTOR), a pathway known to play key roles in cell growth described in detail in section 1.6.1.

The positive effects of growth hormone and IGF-1 on skeletal muscle and bone is well known. Individuals with growth hormone deficiency have low bone mineral density and lean body mass [209]. When commenced on growth hormone therapy, these individuals report improved quality of life, in addition to improvements observed in body composition with gains in lean body mass and loss in fat mass. Furthermore enhanced exercise capacity at the 12 months follow up and improvements in lipid profile have also been observed [210, 211].

IGF-1 levels decline with age in healthy individuals [212, 213]. This phenomenon is thought to contribute further to sarcopenia of ageing. In view of this observation, trials using recombinant growth hormone therapy in the older population were performed to assess its efficacy in skeletal muscle growth and performance. Despite an increase in lean body mass [214] improvements in muscle strength was not observed [215, 216]. Furthermore, reports of side effects (arthralgia, fluid retention and carpal tunnel syndrome) [217] with its use in the older population continues to restrict its role as a potential therapeutic option for sarcopenia.

In the IGF system, the insulin growth factor binding proteins (IGFBPs) are also gaining interest in terms of its role in cell growth. Six IGFBPs have been identified, IGFBP-3 being the most abundant [207, 218]. While the role of the IGFBPs was traditionally thought to prolong the half-life of IGF-1, recent literature now report that the IGFBPs can potentiate and inhibit the effects of IGF-1 [207].

IGFBP3 in particular has been shown to potentiate both IGF-1 and epidermal growth factor (EGFR) signalling [219], another pathway involved in cell proliferation and differentiation. Conversely IGFBP3 can also interact with SMAD2/3 in the TGF-β pathway via activation of TGF-β receptors or nuclear translocation [220] therefore antagonizing cell growth. The SMAD (homologues of the Drosophilia protein, mothers against decapentaplegic) proteins

are a group of intracellular modulators in TGF-β signalling. Myostatin, a member of the TGF-β family (discussed in section 1.6.2) is thought to activate SMAD2/3 resulting in muscle fibrosis [221] and subsequent atrophy.

Although the anti-proliferative effects of IGFBP3 have been explored in oncology research [222], its role in skeletal muscle regulation have not been extensively studied. Thus understanding the cross-talk between IGFBP3, SMAD 2/3 signalling and its effects on the skeletal muscle would be important in understanding muscle homeostasis.

1.5.3 Ghrelin

In 1999 Kojima and colleagues [223] discovered an endogenous ligand which stimulates the release of growth hormone from the anterior pituitary gland. Because of this property, this ligand was subsequently called 'ghrelin'. Its name originates from the Proto-Indo-European word 'ghre', root word for 'grow' [224]. Secreted by the stomach, ghrelin levels rises with starvation or restricted food intake and drops after meals [225]. Although predominantly recognised as a 'hunger hormone', emerging evidence reveal ghrelin has multiple actions ranging from glucose regulation, brown fat metabolism, vasodilatation and cardiac contractility [224], and protection against skeletal muscle atrophy [226, 227].

In view of these observations, anamorelin (a ghrelin receptor agonist) was trialled in patients with cancer cachexia. Despite improvements in lean body mass and grip strength [228], subsequent phase 3 trials did not show any increase in muscle strength [229]. In the older population, the use of ghrelin mimetic resulted in increased fat free mass but no improvement in muscle strength or function [230]. In contrast lean body mass and tandem walk did improve with the use of capamorelin [231] at 12 months. Further large-scale studies are required to determine if the use of ghrelin mimetics consistently shows benefits in terms of improvement on muscle strength, gait speed and reduced disability.

1.5.4 Insulin

Insulin is a hormone secreted by the pancreatic beta cells in the islets of Langerhans with an essential role in glucose homeostasis. It increases intracellular glucose uptake through glucose transporter 4 (GLUT 4) and promotes glycogen synthesis via glycogen synthase, an enzyme involved in the conversion of glucose to glycogen stores.

The insulin receptor belongs to the tyrosine kinase receptor family and is structurally similar in homology to the IGF receptor [232] containing pairs of extracellular alpha and transmembrane beta subunits [219]. As a result of this feature, cross-talk in signalling occurs between both IGF-1 and insulin with these receptors. Although IGF-1 and insulin binds strongly to their respective receptors, insulin at supraphysiological levels can bind to the IGF-1 receptor; and conversely IGF-1 to insulin receptors at lower affinity [219, 232].

Binding of insulin to the insulin receptor results in a conformational change and recruitment of insulin receptor substrate 1 (IRS1). This results in several outcomes. Firstly there is phosphorylation of protein kinase B (AKT) and subsequent mTOR activation [233, 234]. Secondly, GLUT 4 is translocated to the plasma membrane to increase glucose uptake and glycogen synthesis by phosphorylation of glycogen synthase kinase 3 (GSK 3) [233]. As the role of GSK 3 is to inhibit glycogen synthase, insulin via GLUT 4 removes the inhibitory effect of GSK 3 on glycogen synthase thus potentiating glycogen synthesis. In view of insulin effects on the activation of AKT and mTOR [234, 235] the role of insulin in muscle regulation have been examined. Studies in animal models reveal insulin increase protein synthesis, however in humans insulin primarily inhibits muscle breakdown [236, 237].

Ageing is associated with increased insulin resistance and muscle loss. Underlying mechanisms proposed to support this observation include a reduction in GLUT 4 levels, reduced muscle sensitivity to insulin [238] and impaired insulin mediated attenuation of muscle proteolysis [239]. Increasing post prandial insulin levels have not been shown to

increase muscle protein synthesis in these individuals [240]. These factors combined with the 'anabolic resistance of ageing' [241] whereby there is an impaired ability to utilise amino acids to increase protein synthesis in response to feeding adds further to the complexity in muscle regulation in this population.

1.5.5 Thyroid Hormone

The thyroid gland produces thyroid hormones which play integral roles in cellular development, growth and metabolism. Clinical manifestations of thyroid disorders include the presence of myopathy occurring in both hypothyroid and hyperthyroid conditions [242]. Emerging evidence now shows the presence of type 2 (D2) and type 3 (D3) iodothyronine deiodinase in skeletal muscle in humans [243]. The role of these deiodinases is to regulate conversion of the prohormone thyroxine (T4) to the active hormone triiodothyronine (T3). While D2 converts T4 to T3, D3 inactivates T3 and T4 [244].

T3 increases the expression of myoblast determination protein 1 (MYOD1) a myogenic transcriptional activator with subsequent satellite cell activation and myogenesis [243]. Furthermore, T3 also promotes a shift in slow Type 1 muscle fibres to fast Type 2 muscle fibres [245].

Increased age is associated with a rise in serum thyroid stimulating hormone (TSH), unchanged T4 levels [246] and a reduction in T3 levels [247]. Whether or not these changes relate to the loss of skeletal muscle with aging remains to be elucidated and requires further exploration.

1.5.6 Parathyroid Hormone

Parathyroid hormone's key role is in the regulation of calcium metabolism. Serum parathyroid hormone levels above 4 picomoles/L with low vitamin D levels have been associated with increased sarcopenia risk based on grip strength (odds ratio 1.71, 95% CI 1.07-2.73) and muscle mass (OR 2.35, 95% CI 1.05-5.28) [248]. Additionally elevated parathyroid hormone levels was associated with increased sarcopenia risk (OR 6.88 95% CI 1.9-9.2) independent of vitamin D levels [249].

Although improved muscle strength has been observed in those with primary hyperparathyroidism following parathyroidectomy [250], administration of parathyroid hormone in animal models has also been shown to accelerate myocyte differentiation and improve muscle strength [251]. Underlying mechanisms to this remains uncertain and further research is needed to explore the relationship between parathyroid hormone and skeletal muscle.

To summarise the relevance between the different hormones and skeletal muscle, serum IGF-1 and testosterone are the major hormones where there are consistent evidence in their anabolic properties on muscle growth. Further studies are needed to further assess the role of the other hormones described. The role of these hormones are explored in Chapter 3, whereby the hormonal profile in a group of older women with hip fracture (a group with increased proportion of sarcopenic individuals) are examined.

1.6 Skeletal muscle regulation: Regulatory Pathways

Muscle homeostasis is dependent on the complex interaction of several different pathways involved in protein synthesis and breakdown. The mechanistic target of rapamycin (mTOR) is extensively researched and has roles in muscle cell development, growth and metabolism. Myostatin a member of the TGF-β family is a known inhibitor of muscle growth, while the striated activator of Rho Signalling (STARS) pathway is gaining increasing interest. These

pathways interact with the ubiquitin proteasome system and autophagy to maintain net protein balance. An imbalance between these interactions results in a net negative balance in muscle growth and subsequently sarcopenia. These pathways will be discussed in the following sections.

1.6.1 Mechanistic Target of Rapamycin (mTOR) pathway.

The mammalian target of rapamycin, now formally known as mechanistic target of Rapamycin (mTOR) [252] plays a pivotal role in cell growth and development. The discovery of mTOR began in 1964, when a compound isolated from bacteria taken from the soils of the South Pacific island of Rapa Nui were observed to have 'anti-fungal, immune suppressive, and anti-tumour properties' [252]. This compound (now identified as rapamycin or sirolimus) was further observed to work with peptidyl prolyl isomerase FKBP12 to inhibit mTOR [252] thus impeding cell growth. In clinical practice, Sirolimus, an mTOR inhibitor is used in transplant medicine as an anti-rejection agent. It is also used as a compound in cardiac drug eluting stents to prevent in stent restenosis. Figure 1.6a (page 64) shows an abbreviated version of the pathways involved in mTOR regulation.

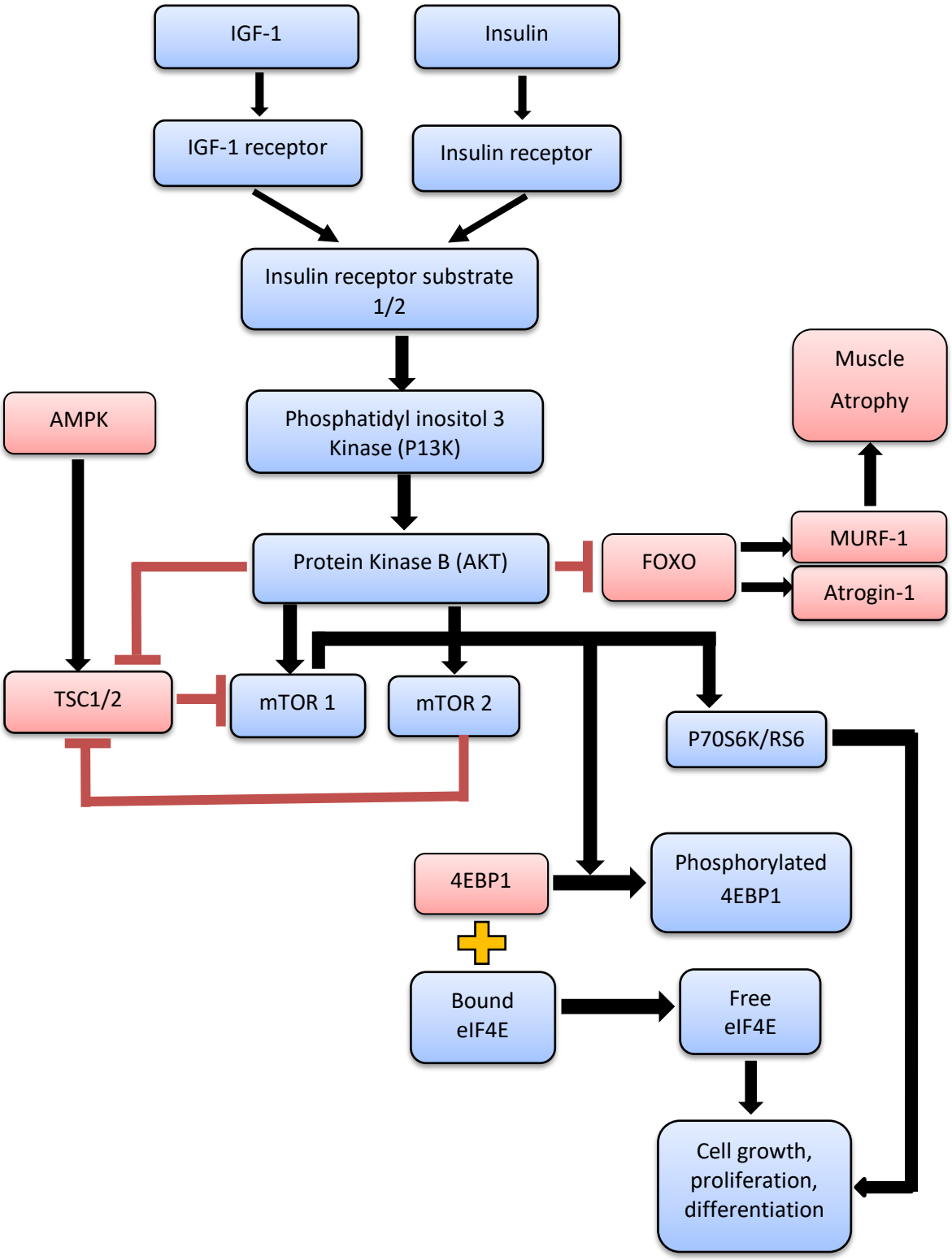
The mTOR pathway is a complex system consisting of a series of downstream signalling cascades. Its activity is triggered by the presence of IGF-1, a hormone produced by the liver with anabolic effects on the bone and skeletal muscle [253]. Serum IGF-1 is predominantly bound in the circulation to IGF binding proteins (IGFBP) due to its short half- life. Following proteolytic cleavage of the IGFBP-IGF-1 complex, release of serum IGF-1 allows it to interact with the IGF receptor present on the cell membrane.

In addition, mTOR is also activated by insulin. As highlighted earlier, insulin and IGF-1 receptors belong to the tyrosine kinase receptor family and therefore share similar structural homology. Although IGF-1 and insulin binds to their respective receptors at high affinity, similarities in their structural homology allows cross talk in signalling between IGF-1 and insulin receptor; and insulin and IGF-1 receptor.

Binding of IGF-1 or insulin to its receptors activate the insulin receptor substrate (IRS) triggering a series of downstream signalling reactions. Six insulin receptor substrates have been identified: IRS1 and IRS2 mostly studied. IRS 1 is thought to play an important role in skeletal muscle through its actions on muscle growth and muscle fibre differentiation, whilst initiating glucose uptake and glycogen synthesis [233]. In contrast the role of IRS 2 in muscle regulation remains uncertain. IRS1 activates phosphatidylinositol 3-kinase (PI3K) resulting in phosphorylation of protein kinase B (AKT) and subsequently mTOR, an intracellular serine/threonine signalling pathway associated with cell growth consisting of 2 complexes: mTORC1, and mTORC2. An abbreviated diagram of mTOR signalling is outlined on the following page in Figure 1.6a on page 65.

Figure 1.6a: Abbreviated diagram of mTOR signalling

Blue background indicates stimulatory (anabolic) pathways, pink background indicates inhibitory (atrophic) pathways, black arrow indicates stimulatory effects, red indicates inhibitory effects



mTORC1 is identified by 3 components; regulator protein associated with mTOR (raptor), mTOR, and mammalian lethal with section 13 protein 8 (MLST8). Inhibitory proteins associated with mTORC1 are proline rich AKT substrate of 40 kilodalton (kda) (PRAS40), and DEP domain containing MTOR interacting protein (DEPTOR) [252].

mTORC2 like mTORC1, also contains MLST8 and mTOR, however it is differentiated by the presence of rapamycin insensitive companion of mTOR (rictor). Its regulatory subunits are DEPTOR, stress activated protein kinase interacting protein 1 (sin1), proctor 1 and proctor 2 [252]. mTORC2 is not affected by the inhibitory effect of rapamycin. However prolonged treatment with rapamycin does reduce mTORC2 signalling likely related to sequestered mTOR bound to rapamycin which limits the formation of new mTORC2 complexes [252, 254]. Both complexes play different roles in cell growth, proliferation and metabolism.

mTORC1 has diverse roles in both anabolic and catabolic pathways as well as lipid and glucose metabolism. Its functions include protein and nucleotide synthesis in addition to inhibition of autophagy and the ubiquitin proteasome system [252]. Increased anabolism via the mTORC1 pathway requires the presence of pro endocrine signals and adequate building blocks like amino acids. In a fed state, activation of mTORC1 results in storage of energy in the liver and muscle [252]. In contrast, in conditions where there is a reduction in cellular energy, hypoxia, or a fasting state, mTORC1 is switched off to conserve energy through the effects of AMP activated protein kinase (AMPK), which activates the tuberous sclerosis complex (TSC), a negative regulator of mTORC1.

Ras homolog enriched in brain (RHEB) is an activator of mTORC1 and is inhibited by the TSC complex, consisting of TSC 1 and TSC2. The TSC complex through its role as a GTPase activating protein on GTPase converts RHEB into its inactive form, thus performing its role as a negative regulator of mTORC1. When AKT is activated, it inhibits TSC allowing subsequent increase in mTOR signalling and activity [252].

Increased protein synthesis occurs through the positive effect of mTORC1 on ribosomal protein p70S6 kinase (P70S6K) or ribosomal protein S6 (RS6), whilst concurrently phosphorylating 4E binding protein1 (4EBP1). Non phosphorylated 4EBP1 conventionally binds to eukaryotic translation initiation factor 4E (eIF4E). The role of eIF4E is to interact with eukaryotic translation initiation factor 4G (eIF4G) to commence protein translation at the 5'- cap of messenger RNA (mRNA). 4EBP1 when bound to eIF4E sequesters eIF4E thus limiting its interaction with eIF4G and therefore restricts protein translation. Activated mTORC1 phosphorylates 4EBP1, allowing phosphorylated 4EBP1 to dissociate from eIF4E. Freed eIF4E recruits eIF4G to subsequently progress towards protein translation. The phosphorylated form of 4EBP1 is considered a marker of increased mTORC1 signalling [255].

mTORC1 is also involved in the regulation of protein turnover through its involvement in the catabolic pathway. In order to maintain cellular health, degradation of faulty proteins and recycling of amino acids occurs via autophagy and the ubiquitin proteasome system [256]. Unc-51 like kinase 1 (ULK1) is involved in autophagy and is usually activated by AMPK. mTORC1 promotes cell growth by phosphorylating ULK1, thus disrupting the interaction between ULK1 and AMPK, resulting in inhibition of autophagy [257].

Although mTORC1 is known for its role in cell growth, mTORC1 also has metabolic effects. It influences sterol responsive element binding protein to promote lipid synthesis and is involved in the induction of glycolysis through the translation of hypoxia inducible factor 1 alpha (HIF1 alpha) [252].

In contrast to mTORC1 (whose role is cell growth and metabolism), the role of mTORC2 is primarily through cytoskeletal remodelling and cell migration through its effects on phosphorylation of members of the protein kinase A, G and C family [252]. mTORC2 is also able to activate AKT resulting in a positive feedback on mTORC1. In animal models, mTORC2 in combination with AMPK was also shown to regulate lipid metabolism [258].

In previous sections the role of AKT in the subsequent activation of mTOR resulting in protein synthesis, metabolic effects and inhibition of the ubiquitin proteasome system and autophagy was described. Apart from activation of the mTOR complex, AKT also inhibits the Forkhead Box O (FOXO) family by phosphorylation and sequestration of FOXO to the cytosol. FOXO is a group of transcriptional factors with multifunctional roles in regulating cell cycle, tumour suppressor function, autophagy, mitophagy and metabolism. Downstream, FOXO activates Muscle Ring Finger Protein-1 (MURF-1) and atrogin-1. These are E3 ubiquitin ligases which polyubiquitinates proteins resulting in the targeting of labelled proteins for subsequent proteolysis [259].

In protein homeostasis FOXO regulates proteasomal degradation and removal of damaged proteins [260, 261]. Increased FOXO activation has been reported in animal models with muscle atrophy [262]. In young healthy humans (mean age 36 ± 4.9 years), Leger examined participants who were subjected to 8 weeks of resistance training [263]. A 10% increase in quadriceps size (compared to baseline) associated with an increase in AKT signalling and reduced FOXO levels were detected at the end of resistance training. These findings further support the role of mTOR and FOXO signalling in muscle regulation. Although acute mTORC1 activation has been shown to increase muscle hypertrophy, prolonged activation of mTORC1 in mice models were also shown to result in severe late onset myopathy. This is thought to be related to impaired autophagy [264] and therefore prolonged activation of mTORC1 may not appear to be beneficial.

1.6.1.1 mTOR in ageing

In recent years increasing interest into the role of mTOR in ageing have led to the discovery that suppression of mTOR signalling in yeast and animal models were associated with longevity [254] and increased lifespan. Increasing age is associated with impaired homeostasis and nutrient sensing, mitochondrial dysfunction and cellular senescence [265]. These factors predispose an older individual to the development of age-related diseases. It is believed that suppression of mTOR contributes to longevity by reducing oxidative stress

and increased clearance of faulty proteins by autophagy [265]. Although it is generally agreed inhibition of mTOR increases lifespan [252] its exact mechanisms in ageing remains uncertain.

In view of mTOR's effect on cell growth, its role in skeletal muscle regulation in ageing was also examined. Sandri [266] evaluated the IGF-AKT-mTOR-FOXO pathway in skeletal muscle from older humans and aged mice. Although an increase in mTORC1 activity was observed in skeletal muscle of older humans (as reflected by increased Ribosomal S6 activity) this increase was modest and failed to reach statistical significance. The authors conclude that findings from their study suggest that loss of muscle with aging was not associated with a down regulation of the IGF-AKT pathway or upregulation of the ubiquitin proteasome system. This therefore reflects the intricacies and complexities around mTOR signalling. The specific aetiology in the mTOR pathway related to muscle loss in ageing remains uncertain and therefore is an area of ongoing research.

1.6.2 Myostatin pathway

Another pathway implicated in muscle regulation is myostatin. Initially discovered by McPherron and colleagues in 1997, myostatin is also known as growth differentiation factor 8 (GDF-8). It is a member of the transforming growth factor beta β (TGF-β) superfamily [267]. Myostatin is predominantly detected in skeletal muscle [267], but can be expressed in cardiac muscle and adipose tissue [268]. In animal models myostatin null mice were 3 times heavier with increased muscle hypertrophy than wild type mice [267].

Myostatin is an extracellular cytokine [269] with both autocrine and paracrine functions. It is secreted by the muscle [270] and exists as a precursor protein in its inactive form consisting of N terminal pro-peptide and a disulphide linked C terminal fragment. Conversion of latent, inactive myostatin into its active form requires the process of two proteolytic events. This

involves the cleavage of the N terminal followed by further cleavage of disulphide linked C terminal dimer by bone morphogenetic protein 1 (BMP-1) [268, 271].

Liberation of the free C terminal dimer (activated form of myostatin) enables it to bind to activin Type IIB receptor (ActRIIB) [268] on the cell membrane resulting in formation of a heterodimer and subsequent recruitment and phosphorylation of activin Type 1 receptors; activin receptor like kinase 4 (ALK4) and activin receptor like kinase 5 (ALK5) [270]. This is followed by phosphorylation of SMAD 2/3, a group of transcriptional factors from the mothers against decapentaplegic (MADs) family.

Phosphorylated SMAD 2/3 recruits SMAD 4 to form the SMAD2/3/4 complex in the cytosol, then travels to the nucleus to commence gene transcription [272] and suppress protein synthesis. Figure 1.6b shows the mechanisms involved in the myostatin signalling pathway.

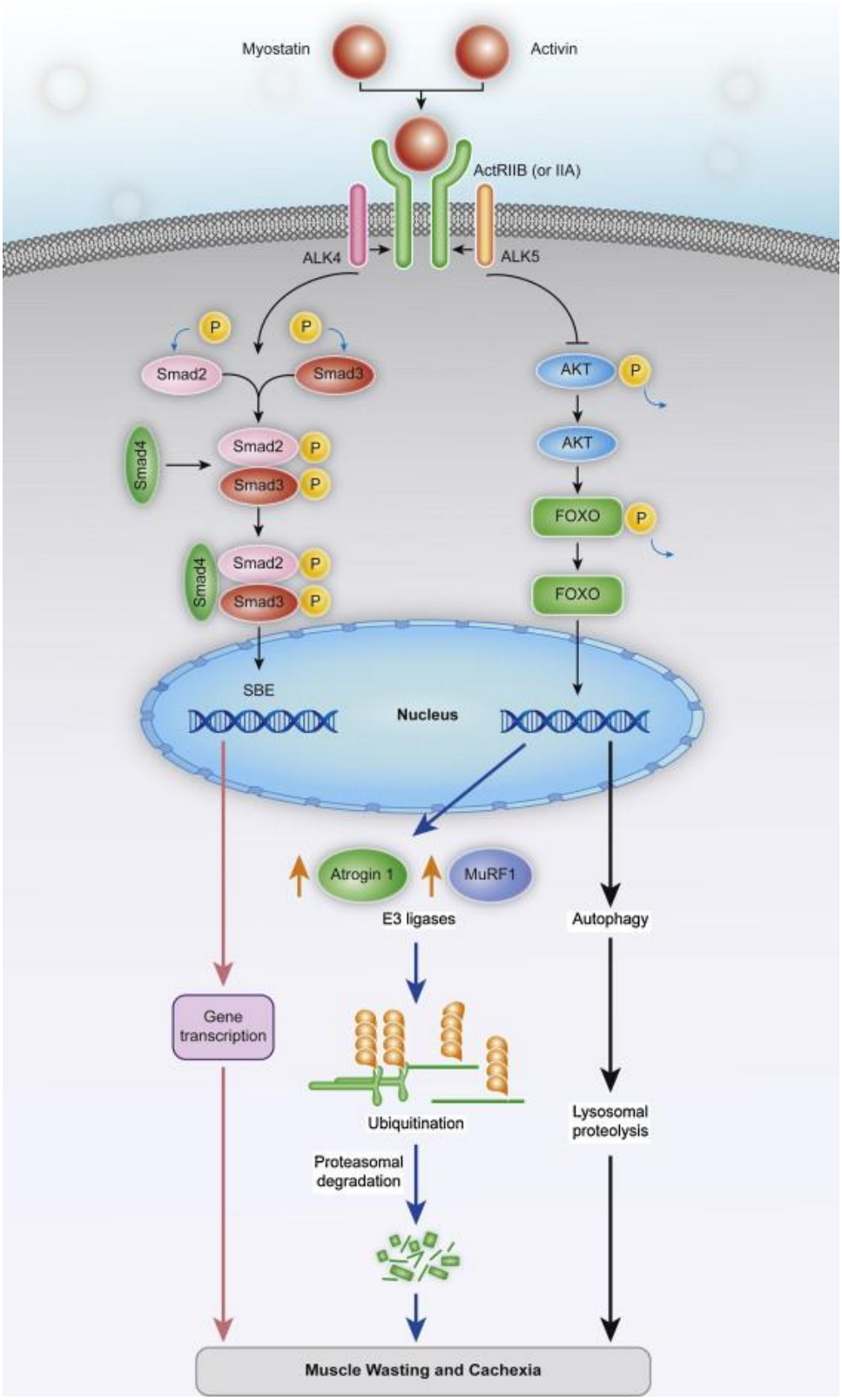


Figure 1.6b: Mechanisms involved in myostatin signalling adapted from Han, 2013. [270]

The SMAD group of proteins are a group of intracellular transcription factors from the mothers against decapentaplegic (MADs) family. It consists of the receptor activated SMAD (including SMAD 2 and 3), common SMAD (SMAD 4) and inhibitory SMAD (SMAD 6,7) [273]. SMAD 7, being an inhibitory transcriptional factor is a negative regulator of myostatin gene expression [274]. As it is upregulated in response to SMAD2/3, SMAD7 also inhibits SMAD 2/3 pathways [273, 275], in which downstream signalling results in a pro fibrotic response [221]. While the upregulation of SMAD 7 in response to androgen have been shown in animal models [206], its role in skeletal muscle regulation in humans have not been examined. Given the link between SMAD 7 and androgens, it is possible that SMAD 7 may have a role to play in muscle homeostasis. This link is examined in Chapter 5 of this thesis.

The negative effect of myostatin on protein synthesis occurs through 2 different mechanisms. The first is the formation of SMAD 2/3/4 complex resulting in induction of FOXO transcription factors with subsequent activation of the ubiquitin proteasome system which includes MURF-1 and atrogin-1/muscle atrophy F box (MAFbx). Both MURF-1 and atrogin-1/MAFbx are E3 ubiquitin ligases which polyubiquitinates proteins resulting in the targeting of these labelled proteins for subsequent proteolysis [259]. The second mechanism in which myostatin inhibits protein synthesis is by inhibiting AKT leading to the removal of the inhibitory effect of AKT on FOXO [270] (Figure 1.6a mTOR signalling pathway).

Other identified roles of myostatin on the muscle are the maintenance of satellite cell in a quiescent state, down regulation of myogenic differentiation factors and inhibition of satellite cell activation by downregulating paired box protein 7 (PAX-7) a transcription factor which regulates myogenesis [276, 277].

There are several inhibitors of myostatin reported in the literature. These include follistatin, growth and differentiation factor associated serum protein 1 (GASP-1), latent TGF beta binding protein 3, and follistatin related gene protein [268, 269, 276]. Of these, follistatin is

an important inhibitor as there have been studies exploring the use of gene therapy in patients with muscular dystrophy.

Follistatin is a glycoprotein and was originally detected from porcine ovarian follicular fluid. It suppresses the production and secretion of follicle stimulating hormone from the pituitary gland, and subsequently was also noted to bind to activin [278] and inhibit the binding of myostatin to ActRIIB [279]. In mice studies using gene therapy, increase muscle mass and hindlimb grip strength were observed in mice overexpressing follistatin [278].

The use of follistatin gene therapy was explored in human participants with Becker Muscular Dystrophy [280] in a phase 1 trial. Participants were monitored for up to 12 months duration. In the 6 study participants (age between 24-30 years old) who had 2 different doses of adeno associated virus delivery of follistatin, 4 study subjects had improvements in their 6 minute walk distance between 28 to 105 metres, whilst no change in distance were observed in the remaining 2 participants. Muscle hypertrophy was also observed. There were no adverse events reported nor were there any changes observed in serum gonadotrophins, testosterone or estrogen levels.

1.6.2.1 Myostatin in ageing

In older humans, elevated serum myostatin levels were reported in those age 76 to 92 years old [281]. However in a separate study comparing older men (age 62-77 years) to a younger cohort (age 21-31 years), messenger RNA (mRNA) analysis using muscle biopsy samples did not detect any difference in myostatin mRNA [282] levels.

The use of myostatin antibody as a potential therapeutic option was examined in a phase 2 trial using myostatin monoclonal antibodies. This was administered to participants age older than 75 years old with falls in the previous 12 months. Increase in appendicular lean mass, stair climb, and gait speed were observed in the treatment group [22]. Reported adverse

events were reaction at the injection sites which were more common in the treatment group (30% vs 9%), although no anaphylaxis was recorded. These findings suggest myostatin antibodies may be another potential treatment option, although its benefit in terms of falls prevention and outcomes on quality of life requires further exploration [22].

In summary, the role of myostatin in muscle regulation is well established from studies observing muscle hypertrophy in animals with the absence of this gene. Like mTOR, the myostatin pathway is a complex signalling system with interactions involving different components. Clinical trials using myostatin antibody appears promising with improved lean body mass and function observed in the treatment group. Further large-scale studies examining the use of this antibody, its long-term effects and benefits in terms of improvement on clinical outcomes and quality of life would provide further insights into its utility as a potential treatment option.

1.6.3 Striated muscle activator of Rho signalling (STARS)

The striated muscle activator of Rho Signalling (STARS) have recently gained interest into its role in muscle regulation. Described in 2002, STARS is an actin binding protein expressed in the heart, skeletal and smooth muscle. It is also known as actin binding Rho activating protein (ABRA) [283].

The sarcomere, a functional contractile unit of the skeletal muscle consists of actin (thin myofilament) and myosin (thick myofilament) (Figure 1.6c). Actin exists in two forms: the globular actin monomer (G actin) and filamentous actin (F actin). Polymerisation of G actin results in the formation and assembly of filamentous F actin, which then interacts with myosin to initiate muscle contraction [284, 285]. STARS together with Ras homolog gene family member A protein (RhoA) promotes gene transcription by polymerising G actin.

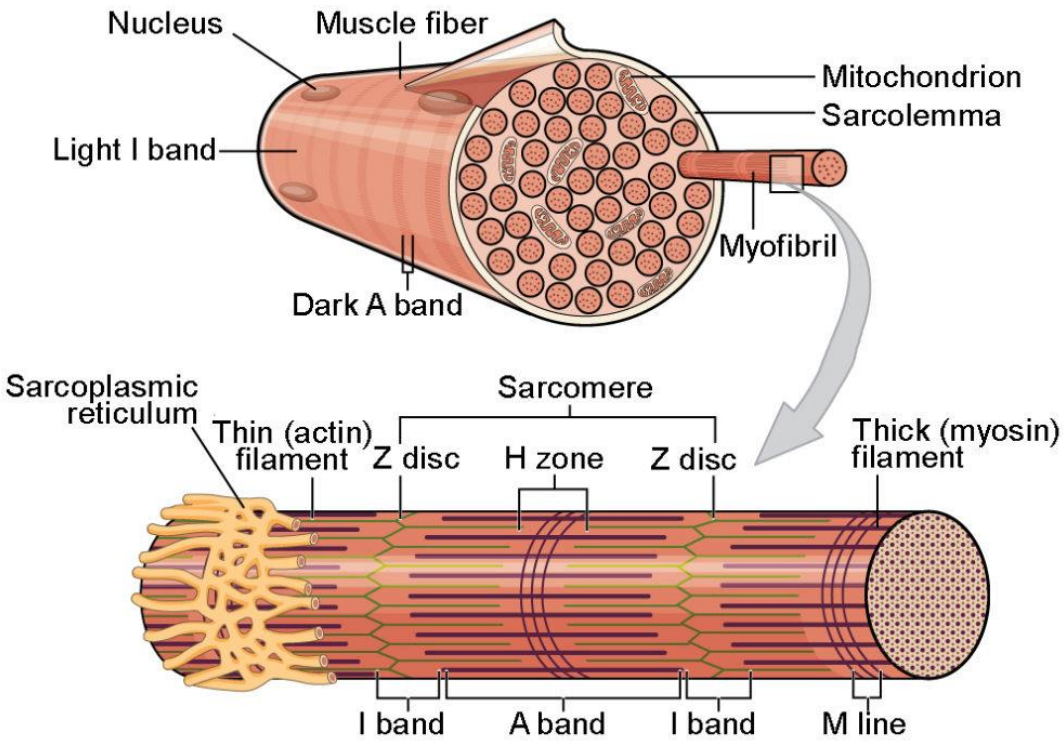


Figure 1.6c: Sarcomere contractile unit of skeletal muscle. Image from OpenStax College Rice University [286]

G actin has an inhibitory effect on myocardin related transcription factor A (MRTF-A) a transcriptional co activator. MRTF-A and serum response factor (SRF) have roles in skeletal muscle differentiation and regulation of the actin cytoskeleton [287]. When bound to G actin, MRTF-A is localised to the cytoplasm and therefore is unable to signal SRF.

STARS when combined with Rho A promotes polymerisation of G actin into F actin and thus by reducing the pool of G actin removes the inhibitory effect of G actin on MRTF-A. Liberated MRTF-A translocates to the nucleus, bind to SRF and commence gene transcription [288] to regulate skeletal muscle growth and repair. This is shown in Figure 1.6d.

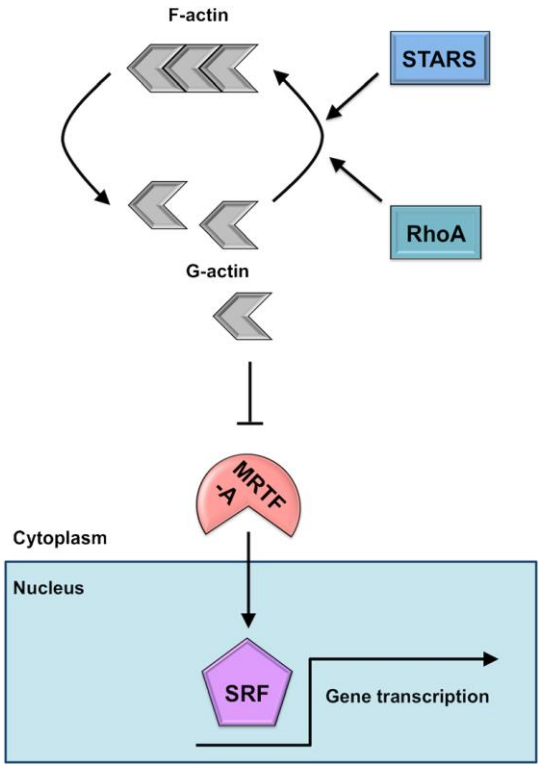


Figure 1.6d: STARS pathway resulting in the subsequent activation of serum response factor. STARS: Striated activator of Rho signalling, Rho A: Ras homolog gene family member A protein, MRTF-A: Myocardin related transcription factor A, SRF: Serum response factor, from Lamon, 2014 [288]

1.6.3.1 STARS in ageing

Reduced STARS expression were observed in aged animal models using mice and pigs [289]. However, studies regarding STARS signalling in older humans are scarce as most studies were predominantly performed in younger participants. A study by Russell [290] compared a total of 20 individuals from the old (60-75 years) and younger (18-25 years) age group who were subjected to resistance exercises. At baseline STARS protein were observed to be lower in the older age group.

Following resistance exercise an increase in messenger RNA (mRNA) expression of STARS and SRF were observed in both groups. However, there was no change recorded in protein expression of these components by western blot analysis. The authors propose this

observation suggest that there is an alternative pathway involved in the post transcriptional process which may include regulation by micro RNA (miRNA) [290].

To summarise, the STARS pathway is another important signalling system involved in skeletal muscle regulation. Unlike mTOR and myostatin the discovery of STARS is fairly recent. Its role particularly in skeletal muscle atrophy in older adults requires further exploration and remains another avenue for research in this field.

1.7 Relationship between muscle, fat and bone

The positive effect of weight on bone is known with prospective studies showing weight gain attenuates bone loss [291, 292]. However it is uncertain if this relationship is related to the effect of skeletal muscle mass or fat mass.

The myocytes, adipocytes and osteocytes share a common origin in the mesenchymal stem cells. Dependant on the transcription factors present, mesenchymal stem cells differentiate into either the adipogenic, myogenic or osteogenic linage. Osteokines released from the bone have been observed to regulate mesenchymal stem cells in fat and skeletal muscle [293]. Additionally, fat mass influence the secretion of hormones from the pancreatic beta cell (amylin, insulin) which also affect bone [294].

In clinical studies assessing the relationship between muscle, fat and bone in post-menopausal women, Reid [292] reported a stronger relationship between bone mineral density (BMD) to weight, body mass index and fat mass when compared to lean mass and BMD. When multiple regression analysis was performed combining fat mass and lean mass in the equation, there was an independent association between fat mass and BMD over the lumbar spine, femoral neck and total body. The same group [295] then showed that changes in fat mass over a 10 year prospective study was predictive of change in BMD.

While these studies support a positive relationship between fat and bone mass, there have also been studies reporting a negative relationship between fat mass and bone [296, 297]. In a study by Kim [297] in pre-menopausal women, fat mass over the truncal and non-truncal region was negatively associated with BMD following adjustment for weight. In the following year, another study was performed in the middle age population (mean 48 years), consisting of both men and women [296]. Body fat percentage was found to negatively correlate with BMD in this group, with the relationship being stronger in men. These findings however were obtained from a Korean population.

Several studies have been performed in the Australian population. In young adults, lean body mass appears to have a stronger relationship with bone [298]. In the Busselton study by Zhu on older middle age individuals, both fat and lean mass was positively associated with bone [299]. However this positive relationship of BMD with fat mass was noted to become negative after a threshold fat mass index of 20 kg/m^2 was reached, suggesting that the positive effects of fat on the bone may be detrimental if weight gain is not accompanied by an increase in lean mass [300]. Given these observations it is likely the relationship between muscle, fat mass and BMD differs dependant on the age group, gender, level of obesity and population. There may also be a threshold in which the gains in BMD with fat mass disappears. Therefore further studies evaluating this relationship particularly in the Australian population would be helpful to determine threshold levels. This is explored in Chapter 2 of this thesis.

1.8 Exercise and muscle

Exercise, particularly resistance exercises, have been shown to be beneficial for the skeletal muscle and currently remains the mainstay of recommended strategies for sarcopenia prevention and management [26-28, 301, 302].

Increase endogenous testosterone signalling [303] and IGF levels [304] have been recorded in response to resistance exercises. At the molecular level, reduced gene expressions of FOXO, MURF-1 and atrogin (pathways involved in muscle atrophy) [305] with increase signalling in the mTOR pathway (pathways involved in muscle growth) [306] have been reported with these exercises.

There are also clinical benefits with resistance exercises. Recent systematic reviews have shown resistance exercise not only prevents muscle loss [302] but is also effective in falls prevention [307, 308]. Preservation of BMD, improvements in balance, muscle mass and strength were observed in trials in post-menopausal women [309]. In the older population, combination of balance training and resistance exercises have been proven to prevent falls [310], improve muscle mass and strength [311]. Furthermore these benefits extend to include nonagenarians living in residential aged care facilities [312]. In those with osteopenia, resistance training from the LIFTMOR trial in a small group of Australian post-menopausal women (n=28) was also shown to be safe with improved function and bone mineral density [313]. Combined with adequate nutrition it is hoped that these measures can attenuate sarcopenia until targeted therapies are developed.

1.9 Summary and Outline of thesis

To summarise the literature review, studies in the field of sarcopenia have risen significantly in the last few years substantiating its clinical relevance and the crucial need to develop treatment options. Prior sections have highlighted the intricacies in signalling pathways and the multifactorial contributors to sarcopenia. Many questions remain unanswered and this thesis aims to fill some of the gaps in this field.

A series of studies were conducted on two different cohorts of women. Cohort 1 is a group of 2041 women age between 40 to 92 years old who were referred to a clinical nutrition service at a tertiary centre for assessment of bone mineral density. A proportion of these women had body composition and anthropometry performed. Results from analysis performed from Cohort 1 are reported in Chapter 2.

A separate analysis was performed on older women in Cohort 2. This is a smaller group of older individuals comprising of a total of forty nine women. In this cohort, twenty women were recruited from the hip fracture group, ten from those awaiting total hip replacement and nineteen from healthy controls. Results from analysis performed in Cohort 2 are reported in Chapters 3, 4 and 5.

Chapter 2 will examine the relationship between skeletal muscle mass, fat mass and bone density, in addition to exploration of the utility of simple anthropometric measures for low skeletal muscle mass in a group of women from Cohort 1.

Chapter 3 examines the hormonal profile in a group of sarcopenic women with hip fracture compared to women awaiting hip replacement and healthy controls (Cohort 2). This chapter aims to identify potential hormonal biomarkers for sarcopenia.

Chapter 4 explores the sex steroid profile including a group of 11-oxygenated androgens produced by the adrenal gland, its relationship to skeletal muscle strength and function and markers of muscle regeneration (as reflected by the presence of central muscle nuclei on histology analysis) in a group of sarcopenic women with hip fracture compared to women awaiting hip replacement and healthy controls (Cohort 2).

Chapter 5 examines the intracellular signalling pathways involved in muscle regulation in muscle biopsy specimens from a group of sarcopenic women with hip fracture compared to women awaiting hip replacement and healthy controls and the relationship to different hormonal biomarkers of sarcopenia (Cohort 2).

It is anticipated that findings from this thesis would provide further insight into the mechanisms of muscle regulation particularly in sarcopenic older women and the use of alternative screening tools for sarcopenia in settings where access to imaging and functional assessment may be limited.

Chapter 2: Analysis of Body composition and anthropometric measures in a cohort of women from a tertiary centre in Southeast Victoria.

2.1 Abstract

This study examines the relationship between skeletal muscle mass and fat mass to bone mineral content and bone density in a group of Australian women in Southeast Victoria, Australia. Simple anthropometric measures as predictors of skeletal muscle mass by DEXA and the potential advantages of the use of leg length in the skeletal muscle index (SMI) were also explored.

Key findings from this study are the positive relationships in both skeletal muscle mass and fat mass to bone mineral content and bone mineral density, independent of age. Calf circumference is a strong predictor of skeletal muscle mass by DEXA. Leg length appears unchanged across age and may be another useful denominator in the SMI for low skeletal muscle mass assessment.

2.2 Introduction

The positive relationship between weight and bone mineral density in post-menopausal women is well established [294, 314, 315]. In those who were obese, weight loss was accompanied by a concurrent loss in bone mineral density [316, 317]. Despite this association, the determinants in this relationship between the components of body composition and bone mass remain uncertain. There is evidence in the literature showing that both lean mass [298, 318] and fat mass [292, 299, 319-322] have a positive relationship with bone density. However there are also conflicting reports of a negative relationship between fat mass and bone [300, 323, 324].

Specifically in the Australian population, low fat mass is an independent risk factor for low bone mass in the Dubbo osteoporosis study [320]. In addition, in the Busselton healthy aging study in women, increase in fat mass index (FMI) was also associated with increase in bone density. The direction in this relationship however was transformed into a negative relationship when a FMI threshold above 20 kg/m^2 was reached [300]. Although both of these studies included older adults, mean age for women enrolled were between 56-68 years. Thus it remains uncertain if these findings are applicable to those older than this age range. In an overseas study by Visser [325] enrolling older adults (mean age 78 years), both muscle and fat mass were positively associated with bone mineral density. Hence one aim of this study was to examine the relationship between lean and fat mass to bone mineral density in a dataset which included older women.

The use of gold standard imaging for low skeletal muscle mass such as MRI and CT are limited by radiation, costs and accessibility. DEXA as an alternative is gaining popularity due to its precision and low radiation. However access to DEXA remains a challenge as it is not readily available at all hospital sites in Victoria. Studies overseas explored the use of simple anthropometric measures as a predictor for skeletal muscle mass by DEXA. Findings from studies on those in the community demonstrates calf circumference correlate well with appendicular lean mass by DEXA [326, 327] and calf muscle on MRI [328]. In addition low calf circumference was also associated with increased frailty, disability and impaired physical function [329, 330].

In Australia, population studies using anthropometry data from height, weight and body mass index (BMI) measurements to estimate skeletal muscle mass have been performed [331, 332]. However, accurate and precise measurement of height may be challenging especially in those with mobility impairment. Given the good correlation between calf circumference to skeletal muscle mass, simple measurements such as limb circumference may be a useful alternative to BMI in estimating skeletal muscle mass.

To our knowledge studies exploring anthropometric measures with limb circumference have not been performed in the Australian population. Given the ease and convenience of limb circumference measurement, we compared anthropometric measures and explored the utility of anthropometric measurements using limb circumference as a predictive tool for skeletal muscle mass assessment in our cohort.

Increase age is associated with a decline in height. Given the use of height as a denominator in the height adjusted skeletal muscle index by Baumgartner (H-SMI), there may be a propensity for this index to underestimate low skeletal muscle mass, particularly in the older population. Hence the third aim in this study was to explore the use of leg length in the skeletal muscle index.

2.3 Aims

- To examine the relationship between skeletal muscle mass to bone mineral content (BMC) and bone mineral density (BMD)

- To examine the relationship between fat mass to bone mineral content (BMC) and bone mineral density (BMD)

- To examine anthropometric measures and its utility as a predictive tool in skeletal muscle mass estimation by DEXA

- To examine the use of leg length as a denominator in the height adjusted skeletal muscle index

2.4 Methods

This study is a secondary analysis of a large dataset belonging to Professor Boyd Strauss who is affiliated with Monash University. Ethics approval for the use of this data was obtained from Monash University Human Research Ethics Committee; Project number 1158.

2.4.1 Participants description

A dataset from 2521 women from South-East Victoria, Australia from a convenience sample were screened. Participants in this study were women who were referred primarily for assessment of bone mineral density to a clinical nutrition service who consented to the use of their data for research analysis. A proportion of these women had body composition and anthropometry measures documented. Sources of referral were from general practitioners and hospital specialty clinics.

Consent, data collection and anthropometric measurements were performed by trained staff, nurses and experienced anthropometer based at the Clinical Nutrition Unit at Monash Health. Bone mineral density and body composition assessment was performed by a trained DEXA technician attached to the unit. Data collected were collated into a secure password-protected electronic database. Although hospital identifiable unit record number were available for some participants in the database, confidentiality of the participants were maintained as the analysis was performed by assessors who had no access to the participant's hospital records.

2.4.2 Anthropometry measurements

Participants had a minimum of 2 hours fast prior and were weighed in light clothing to the closest 1 kilogram. Standing and sitting height were measured to the closest 0.1 centimetre with a Holtain stadiometer as outlined by Lohman [333]. Leg length was calculated by subtracting sitting from standing height.

Limb circumference measurements was performed using the following landmarks and methods provided by Lohman [333]. Measurements were performed in triplicate and an average was taken as the final reading.

1. Mid upper arm circumference: At the level midpoint between the acromial process and elbow flexed at 90 °.
2. Gluteal circumference: At the buttocks at the level of the greater trochanter.
3. Thigh circumference: At the mid-point level between the inguinal crease to proximal border of the patella.
4. Calf circumference: At the maximum circumference perpendicular to the long axis of the calf.

2.4.3 Body composition and bone mineral density by DEXA

Body composition and bone mineral density assessment was performed using the Lunar Prodigy DEXA machine, between 2004-2012, by a trained DEXA technician. Calibration measures with a phantom were performed for bone mineral density daily, and body composition weekly. Data for lean, fat mass and bone mineral density were obtained using the General Electric Lunar Prodigy Software, version 3.6 (Madison, WI). The coefficient of variation for the lumbar spine BMD, lean and fat mass phantom was 0.51%, 3% and 3.11% respectively. With each update in DEXA software, data acquired from older versions were reanalysed for consistency with the most updated software.

Skeletal muscle index and fat mass index were calculated using the following formula:

Skeletal muscle mass index (SMI): Appendicular lean mass [arms and legs lean tissue mass(kg)]/ height^2 (m^2)

Fat mass index (FMI): Fat mass (kg) / height^2 (m^2).

Fat mass in this study is calculated as whole body fat mass.

2.4.4 Selection of participant for analysis

A total of 2521 women were screened. This was a secondary analysis on a dataset obtained from women who were referred to a body composition laboratory for assessment of their nutritional state or bone mineral density. 87 women with body mass index ≤ 18 kg/m^2 were excluded from the analysis as it was felt that this group was not a true reflection of the population assessed. Another group of 393 women were excluded from the analysis due to the absence of DEXA skeletal muscle mass data (n=344), or significantly lowered skeletal muscle mass by DEXA ≤ 8kg (n=49). In the 2041 women analysed, anthropometric data was available from 112 women and leg length data from 100 women.

To determine if the relationship between lean and fat mass to bone were applicable to older women, and those with FMI > 20 kg/m^2, a subset analysis was performed. Due to the absence of DEXA SMM data in those with FMI > 20kg/m^2, 135 women from the initial 393 women who were excluded were used for this analysis, using appendicular lean mass as an alternative to DEXA skeletal muscle mass for analysis. Analysis for leg length in the use of the skeletal muscle mass index was performed on 137 women in whom leg length measurements were available. Figure 2.4.4a outlines the participant selection for the specific analysis.

2.4.5 Statistical analysis

Analysis was performed using SPSS version 26 (IBM, Chicago). Parametric data were expressed as means ± standard deviation (SD), whilst non-parametric data were expressed as median (interquartile range). A p value < 0.05 were selected to indicate statistical significance.

Reporting of body composition and anthropometric data were presented with the women divided into younger (age < 60 years) and older (age > 60 years) groups. The data was

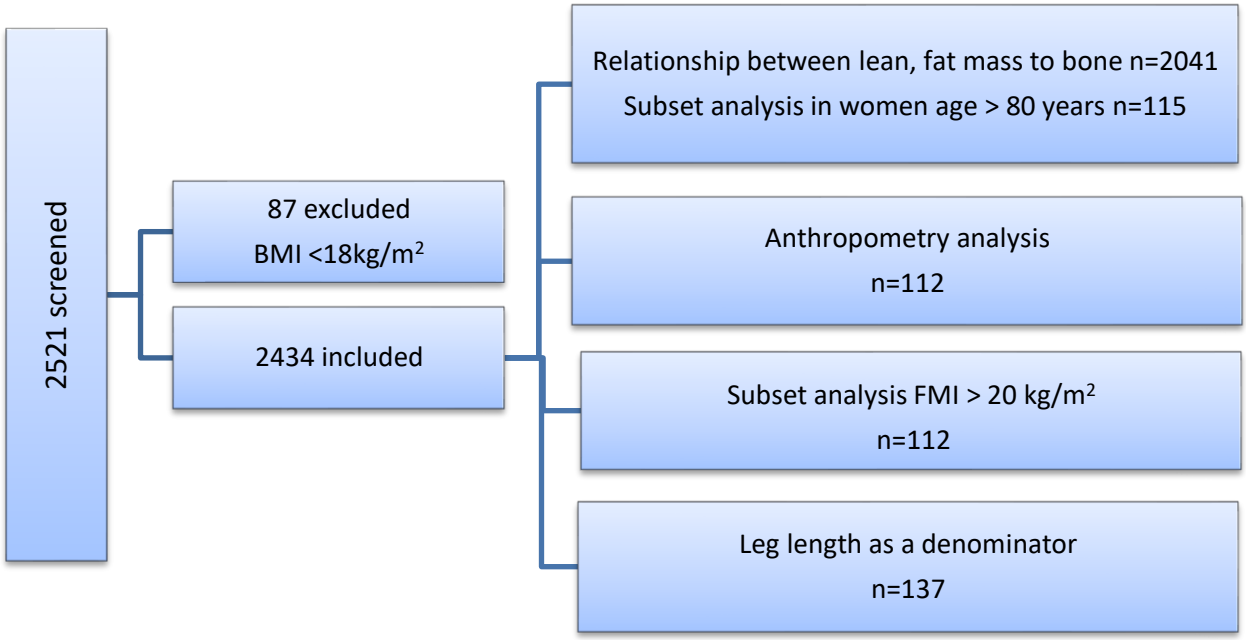
presented in this manner as we wanted to explore the utility of leg length as a denominator in the SMI. We hypothesize younger women have higher skeletal muscle mass and are taller than older women. Groupwise comparison was performed using student's T test for parametric data, Mann Whitney U test for non-parametric data and Chi square test for categorical data. Women in each group were categorised into 'low' and 'normal' skeletal muscle mass using height adjusted SMI cut points <5.5kg/m^2 from the EWGSOP 2 criteria [34] to compare the prevalence of low skeletal muscle mass in each group.

The relationship between skeletal muscle mass and fat mass to BMC and BMD were assessed as continuous variables. Univariate regression analysis was initially performed to determine the strength of the relationship, followed by multiple regression analysis adjusting for age with BMC and BMD as the outcome variable.

Anthropometric measures were assessed in a univariate regression analysis to determine each variable which were significantly associated with DEXA SMM. Multiple regression analysis was then performed to determine the strongest anthropometric measure as a predictor of skeletal muscle mass.

In the assessment of leg length as a denominator in the SMI, scatter plots were first created to assess the relationship between height and leg length to age. A comparison of height adjusted SMI (H-SMI) and leg length adjusted SMI (LL-SMI) was performed comparing groups of younger to older women.

Figure 2.4.4a Selection of participant for analysis (Cohort 1)



2.5 Results

2.5.1 Demographics

Table 2.5.1a provide a list of the participants' medical conditions and indications for referral. The majority of these women were referred for osteoporosis screening, followed by premature menopause and a history of previous fracture. Median age was 58 (51, 67) years old, range 40-92 years (Table 2.5.2a). Women in this cohort were divided into 2 groups for comparison: Group 1 (age 40-60 years, n=1163) and Group 2 (age 61-100 years, n=878). Younger women were taller and had a lower body mass index than older women.

Table 2.5.1a: Medical condition and indication for referral

Category	**Frequency**	**Percent (%)**
Osteoporosis Screening	581	23.9
Premature menopause	385	15.8
Previous fracture	291	12
Breast cancer	284	11.7
Steroid use	206	8.5
Others (Hypogonadal state, anticonvulsant use)	179	7.4
Rheumatological condition	117	4.8
Known Osteoporosis	98	4
Thyroid, parathyroid abnormalities	94	3.8
Chronic Kidney Disease	53	2.2
Post-transplant	45	1.8
Coeliac Disease	34	1.4
Diabetes	22	0.9
Chronic liver disease	23	0.9
Turners Syndrome	15	0.6
Bariatric Surgery	7	0.3

Table 2.5.2a: Demographics and body composition data for study participants

Category	**Whole Population** n=2041	**Group 1** Age 40-60 years n=1163 (57%)	**Group 2** Age 61-100 years n=878 (43%)	**p value**
Age (years)	58 (51,68)	52 (47,56)	70 (65,76)	<0.01
Height (cm)	159.4 ± 7.2	160.9 ± 6.9	157.3± 7.1	<0.01
Weight (kg)	65.8 (58.0, 74.7)	65.4 (57.9, 75.3)	66.1 (58.0, 74.1)	0.70
Body mass index (kg/m^2)	25.9 (22.9, 29.4)	25.3 (22.6, 28.9)	26.9 (23.5, 29.8)	<0.01
Body composition data				
DEXA SMM (kg)	17.3 (15.5,19.2)	17.8 (16.0, 19.5)	16.7 (15.0, 18.5)	<0.01
DEXA ALM (kg)	15.5 (14.0,17.1)	15.8 (14.2, 17.3)	15.3 (13.6, 16.6)	<0.01
DEXA fat mass (kg)	25.5 (19.6, 32.5)	24.7 (19.2,32.3)	26.4 (20.3, 32.8)	0.15
DEXA FMI (kg/m^2)	10.1 (7.7,12.8)	9.7 (7.4, 12.5)	10.7 (8.2, 13.1)	<0.01
Bone density data				
DEXA BMC (kg)	2.2 (2.0, 2.5)	2.3 (2.1, 2.6)	2.1 (1.8, 2.3)	<0.01
DEXA total body BMD (gm/cm^2)	1.1 (1.0,1.2)	1.1 (1.0,1.2)	1.0 (0.9,1.1)	<0.01
DEXA femoral neck BMD (gm/cm^2)	0.8 (0.7, 0.9)	0.90 (0.8, 0.9)	0.7 (0.7, 0.8)	<0.01
DEXA femoral neck T score	-1.0 (-1.8, -0.2)	-0.6 (-1.3, 1.0)	-1.6 (-2.2, -0.8)	<0.01
Skeletal muscle index comparison				
Height adjusted SMI (kg/m^2) (H-SMI)	6.14 ± 0.8	6.13 ± 0.73	6.16 ± 0.81	0.24
'Low' skeletal muscle mass (yes/no) by EWGSOP 2, n (%) †	420 (20.6%)	239 (20.6%)	181 (20.6%)	0.97

DEXA SMM: DEXA skeletal muscle mass, DEXA ALM: DEXA Appendicular Lean Mass, DEXA SMI: DEXA Skeletal muscle mass index, DEXA BMC: DEXA Bone Mineral Content, DEXA total body BMD: DEXA total body bone mineral density, DEXA femoral neck BMD: DEXA femoral neck bone mineral density, † 'low' skeletal muscle mass defined by H-SMI < 5.5 kg/m^2 as per EWGSOP 2 criteria

2.5.2 Groupwise comparison: Body composition, BMC, BMD by DEXA (Table 2.5.2a)

Older women had significantly lower skeletal muscle, appendicular lean mass and higher fat mass index than younger women. BMC, BMD and femoral neck T scores were significantly lower in older women who were also more osteopenic than the younger age group.

Although younger women had higher skeletal muscle mass than older women, a comparison using the conventional height adjusted skeletal muscle index (H-SMI) shows no significant difference between groups. Prevalence of low skeletal muscle mass by EWGSOP 2 criteria were similar between both groups (20.6% vs 20.6%, p=0.97).

2.5.3 Relationship between lean, fat mass to BMC and BMD

To assess the relationship between lean and fat mass to BMC and BMD, scatter plot (lean mass against BMC/BMD, fat mass against BMC/BMD) was first created to assess the trend. Lean mass and fat mass were positively associated with BMC and BMD (Figures 2.5.3a-d).

Univariate regression analysis was performed followed by multiple regression analysis. In the age adjusted model, both lean and fat mass were positively associated with BMC and BMD (Tables 2.5.3a-b). Multiple regression analysis demonstrates (Table 2.5.3c) lean and fat mass remained significantly associated with BMC and BMD independent of age. The association between these variables to BMC were stronger (r^2= 0.51) when compared to BMD (r^2=0.37).

Figure 2.5.3a: Scatter plot DEXA skeletal muscle mass (kg) to DEXA bone mineral content (g)

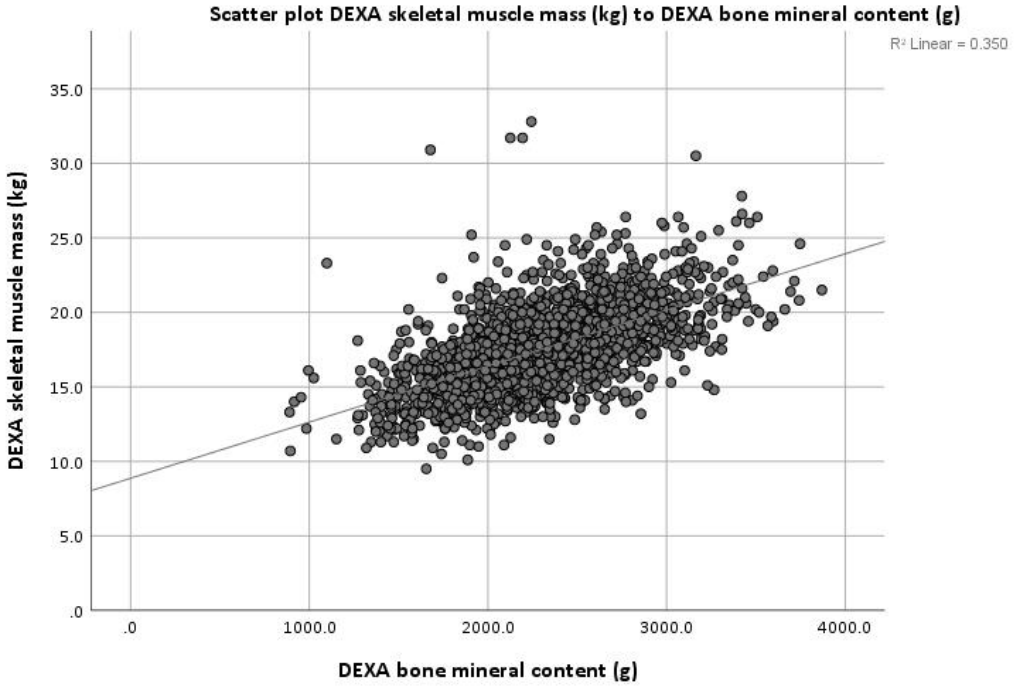


Figure 2.5.3b: Scatter plot DEXA skeletal muscle mass (kg) to DEXA total body bone mineral density (g/m^2)

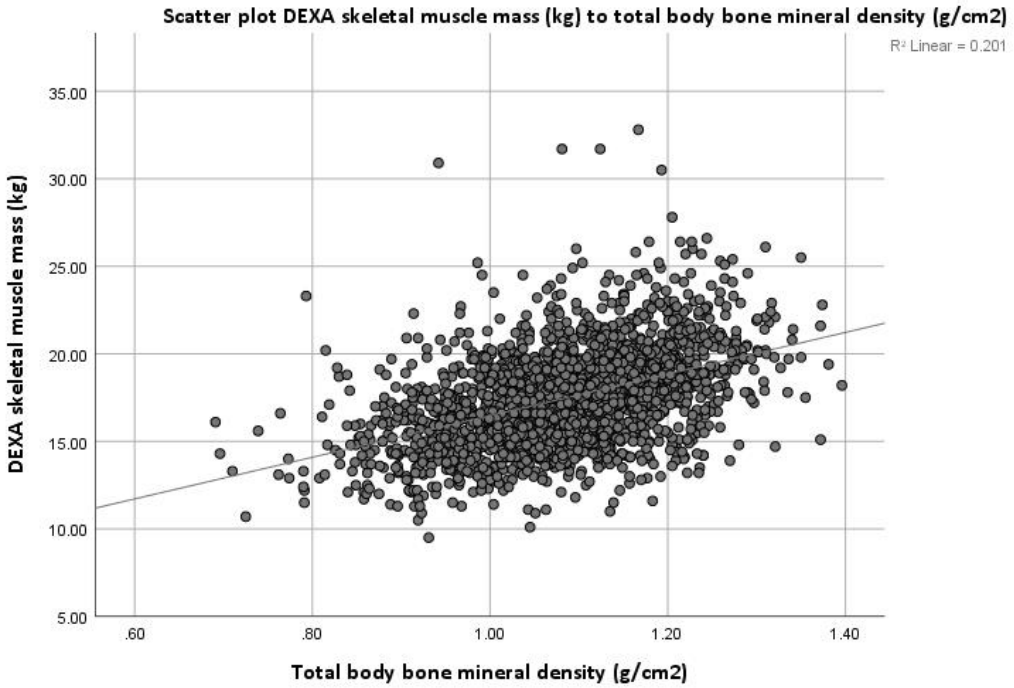


Figure 2.5.3c: Scatter plot DEXA fat mass (kg) to DEXA bone mineral content (g)

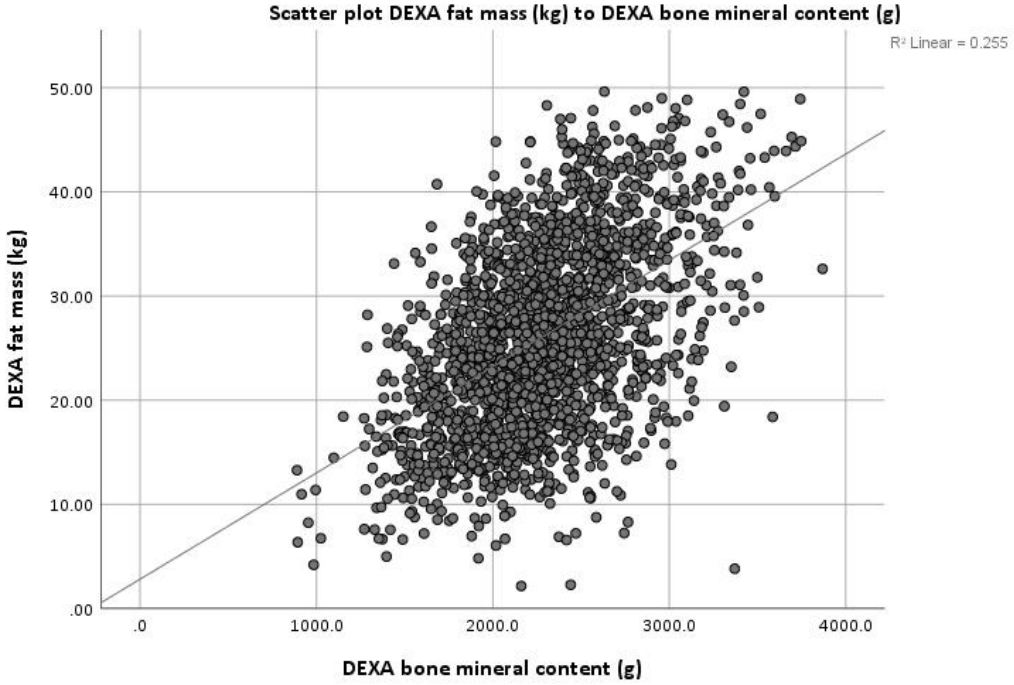


Figure 2.5.3d: Scatter plot DEXA fat mass (kg) to DEXA total body bone mineral density (g/cm^2)

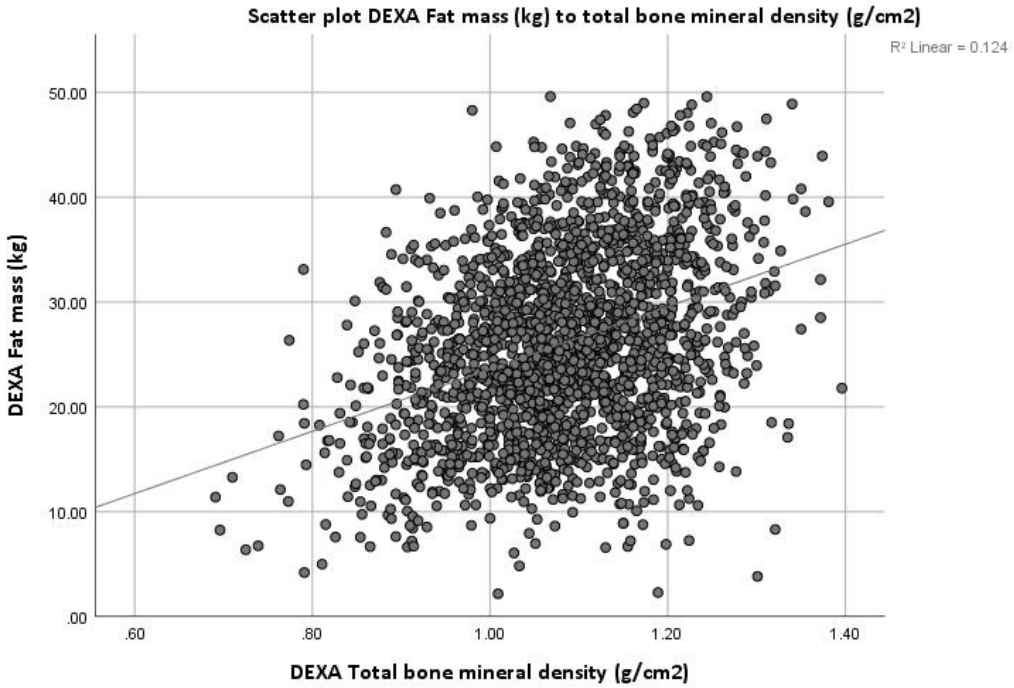


Table 2.5.3a: Linear regression analysis with bone mineral content (g) as outcome variable

Independent variable	**r^2**	**Beta (95% CI)**	**p value**
DEXA skeletal muscle mass (kg)	0.350	93.10 (87.59, 98.60)	<0.01
DEXA fat mass (kg)	0.255	24.97 (23.11, 26.82)	<0.01
Age adjusted model			
Independent variable	**r^2**	**Beta (95% CI)**	**p value**
DEXA skeletal muscle mass (kg)	0.407	84.27 (78.87, 89.67)	<0.01
DEXA fat mass (kg)	0.389	24.92 (23.24, 26.60)	<0.001

Table 2.5.3b: Linear regression analysis with total body bone mineral density (g/cm^2) as outcome variable

Independent variable	**r^2**	**Beta (95% CI)**	**p value**
DEXA skeletal muscle mass (kg)	0.201	0.017 (0.015, 0.018)	<0.01
DEXA fat mass (kg)	0.124	0.004 (0.004, 0.005)	<0.01
Age adjusted model			
Independent variable	**r^2**	**Beta (95% CI)**	**p value**
DEXA skeletal muscle mass (kg)	0.323	0.014 (0.012, 0.015)	<0.01
DEXA fat mass (kg)	0.320	0.004 (0.004,0.005)	<0.01

Table 2.5.3c: Multiple regression analysis model with bone mineral content (g) and bone mineral density (g/cm^2) as outcome variable

Relationship to bone mineral content (g)			
Independent variable	**r^2**	**Beta (95% CI)**	**p value**
DEXA skeletal muscle mass (kg)	0.511	61.55 (56.20, 66.91)	<0.01
DEXA fat mass (kg)		17.38 (15.74, 19.02)	<0.01
Age (years)		-10.55 (-11.74, -9.35)	<0.01
Relationship to bone mineral density (g/cm^2)			
Independent variable	**r^2**	**Beta (95% CI)**	**p value**
DEXA skeletal muscle mass (kg)	0.375	0.010 (0.008, 0.011)	<0.01
DEXA fat mass (kg)		0.003 (0.003, 0.003)	<0.01
Age (years)		-0.003 (-0.004, -0.003)	<0.01

Relationship between lean and fat mass to BMC and BMD in women older than age 80 years

To determine if the positive relationship between lean and fat mass to bone were applicable to older women, subset analysis was performed in a group of women age 80 years old and above from the population. Data from 115 women were analysed. In a multiple regression analysis, the positive relationship between lean, fat mass and BMC/BMD were maintained after adjusting for age in women over 80 years (Table 2.5.3d).

Table 2.5.3d: Multiple regression analysis with bone mineral content (g) and bone mineral density (g/cm^2) as outcome variable, subset analysis of women age > 80 years

Relationship to bone mineral content (g)			
Independent variable	**r^2**	**Beta (95% CI)**	**p value**
DEXA skeletal muscle mass (kg)	0.39	39.04 (12.35, 65.73)	<0.01
DEXA fat mass (kg)		18.69 (11.11, 26.26)	<0.01
Age (years)		-24.51 (-45.19, -3.83)	0.02
Relationship to bone mineral density (g/cm^2)			
Independent variable	**r^2**	**Beta (95% CI)**	**p value**
DEXA skeletal muscle mass (kg)	0.287	0.008 (0.000,0.016)	0.05
DEXA fat mass (kg)		0.004 (0.001,0.006)	<0.01
Age (years)		-0.010 (-0.016, -0.004)	<0.01

Relationship between appendicular lean mass, fat mass to BMC/BMD in those with FMI > 20kg/m^2 (n=135)

To explore the relationship between lean and fat mass in those with FMI > 20kg/m^2 further analysis was performed in women with FMI > 20kg/m^2. In the original analysis on the 2041 women, none had FMI > 20kg/m^2. Thus 135 women from those who had missing DEXA SMM were selected for this analysis. Due to the absence of DEXA SMM, appendicular lean mass were used as an alternative. Median age in this group was 53 (45,63), range 40-87 years. Median FMI was 22.1 kg/m^2 (20.8, 23.8).

In a multiple regression analysis, appendicular lean mass (ALM) and fat mass were positively associated with BMD independent of age (Table 2.5.3e), although only fat mass was a significant determinant of bone mineral content. However ALM and height adjusted ALM (SMI) had a stronger relationship to bone density than FMI. We did not find evidence to support a negative relationship between fat mass and BMD in women with FMI > 20 g/m^2.

Table 2.5.3e: Multiple regression analysis with bone mineral content (g) and bone mineral density (g/cm^2) as outcome variable, subset analysis in those with FMI > 20kg/m^2

Relationship to bone mineral content (g)			
Independent variable	**r^2**	**Beta (95% CI)**	**p value**
DEXA ALM (kg)	0.21	14.46 (-11.33, 40.66)	0.27
DEXA fat mass (kg)		24.45 (11.81, 37.09)	<0.01
Age (years)		-4.93 (-12.04, 2.17)	0.17
Relationship to bone mineral density (g/cm^2)			
Independent variable	**r^2**	**Beta (95% CI)**	**p value**
DEXA ALM (kg)	0.34	0.010 (0.005, 0.014)	<0.01
DEXA fat mass (kg)		0.004 (0.001,0.006)	<0.01
Age (years)		-0.002 (-0.003, 0.000)	<0.01
Relationship to bone mineral density (g/cm^2), analysis using FMI			
Independent variable	**r^2**	**Beta (95% CI)**	**p value**
DEXA ALM (kg)	0.31	0.013 (0.009, 0.017)	<0.01
DEXA FMI (kg/m^2)		0.006 (-0.001, 0.012)	0.10
Age (years)		-0.002 (-0.003, -0.001)	<0.01
Relationship to bone mineral density (g/cm^2), analysis using SMI (height adjusted), FMI			
Independent variable	**r^2**	**Beta (95% CI)**	**p value**
DEXA height adjusted ALM = SMI (kg/m^2)	0.207	0.027 (0.012, 0.042)	<0.01
DEXA FMI (kg/m^2)		0.005 (-0.003, 0.012)	0.20
Age (years)		-0.003 (-0.004, -0.001)	<0.01

DEXA ALM: DEXA appendicular lean mass, DEXA FMI: DEXA Fat mass index, SMI: Skeletal muscle index

2.5.4 Exploration of anthropometric variables as predictors of skeletal muscle mass (n=112)

Data for anthropometric assessment were available from 112 women. Groupwise comparisons are summarized in Table 2.5.4a. Mid upper arm, thigh and calf circumference were significantly higher in younger women compared to older women ($p<0.05$). Multiple regression analysis was performed between anthropometric variables to skeletal muscle mass as the dependant variable. As there was a significant correlation between mid-upper arm circumference and gluteal circumference ($r=0.73$), and between thigh and calf circumference ($r=0.84$), gluteal and thigh circumference was excluded from the multiple regression analysis. In the model adjusted for age, calf circumference was the strongest predictor of DEXA skeletal muscle mass (Table 2.5.4b).

To determine if a specific cut off value for calf circumference can be used to predict low skeletal muscle mass by DEXA, a receiver operating curve (ROC) was performed using EWGSOP 2 SMI cut point < 5.5 kg/m^2 to define low skeletal muscle mass in women. A selected calf circumference of < 35.6 cm was associated with a sensitivity of 88.6%, specificity of 54.5%. Area under the curve (AUC) was 0.829, p value <0.001, 95% CI 0.749-0.908 (Figure 2.5.4a). In contrast AUC for mid upper arm circumference was 0.687, p value 0.002, 95% CI 0.588-0.785, further supporting calf circumference as a better predictor of DEXA skeletal muscle mass in this cohort.

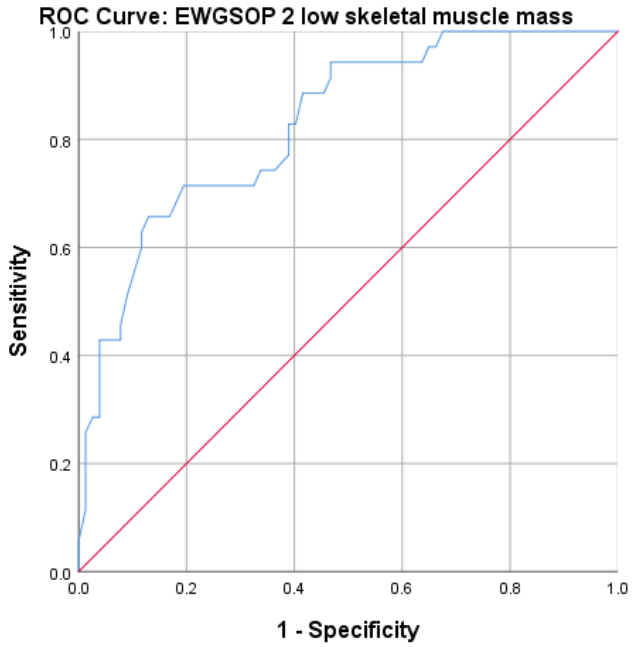
Table 2.5.4a: Anthropometric data comparison between groups

Category	**Whole Population** n=112	**Group 1** (40-60 years) n=80	**Group 2** (61-100 years) n=32	**p value**
Mid upper arm circ (cm)	28.0 (25.4,32.0)	28.6 (25.7,32.7)	27.0 (24.1,30.5)	0.03
Gluteal circ (cm)	97.5 (91.0,105.5)	99.0 (92.0,106.0)	95.0 (90.0,105.0)	0.47
Thigh circ (cm)	48.4 (44.5,53.9)	51.0 (45.9,55.6)	45.0 (42.6,51.0)	<0.01
Calf circ (cm)	34.6 (32.5, 37.9)	35.4 (33.5,38.4)	33.0 (31.4, 35.1)	<0.01

Table 2.5.4b: Multiple regression analysis between anthropometric variables to skeletal muscle mass on DEXA (kg)

Independent variable	r^2	Beta (95% CI)	p value
Age	0.434	-0.016 (-0.052,0.020)	0.39
Mid upper arm circumference		0.020 (-0.088,0.129)	0.71
Calf circumference		0.478 (0.335,0.621)	<0.01

Figure 2.5.4a: ROC curve for calf circumference using EWSGOP 2 criteria for low skeletal muscle mass.



2.5.5 Exploration of leg length as a denominator in the skeletal muscle index (n=137)

As height declines with age, the use of leg length as a denominator in the skeletal muscle index was explored. Younger women were comparatively taller than older women although height did not reach statistical significance. There was no difference observed in leg length (Table 2.5.5a). To examine this finding further, a scatter plot was created using height and leg length against age (Figure 2.5.5a). There was a trend towards lower height in older women, while leg length appears unchanged across the age groups.

As there was no significant difference observed in leg length, a scatter plot using H-SMI and LL-SMI was performed. A decline in LL-SMI compared to H-SMI appeared more obvious when visualised in a scatter plot. In the comparison of H-SMI and LL-SMI between age groups, younger women had significantly higher skeletal muscle mass index with both indices. (Table 2.5.5a).

Table 2.5.5a Groupwise comparison height, leg length and SMI

Category	**Whole Population** n=137	**Group 1** (40-60 years) n=106	**Group 2** (61-100 years) n=31	**p value**
Height (cm)	161.4 ± 0.5	162.0±5.6	157.4±8.4	0.12
Sitting height (cm)	85.4 (82.6, 87.5)	85.9 (83.1,88.4)	83.6 (80.9,85.7)	<0.01
Leg length (cm)	76.5 (73.7, 79.0)	76.6 (73.7,79.0)	75.6 (73.7,79.1)	0.92
H-SMI	6.4 (5.6, 7.6)	6.6 (5.7, 7.9)	6.2 (5.4, 6.8)	0.02
LL-SMI	29.3 ± 0.6	30.2 ± 7.5	26.4 ± 5.2	<0.01

H-SMI: Height adjusted skeletal muscle index (kg/m^2), LL-SMI: Leg length adjusted skeletal muscle index (kg/m^2)

Figure 2.5.5a: Scatter plot showing height and leg length across age

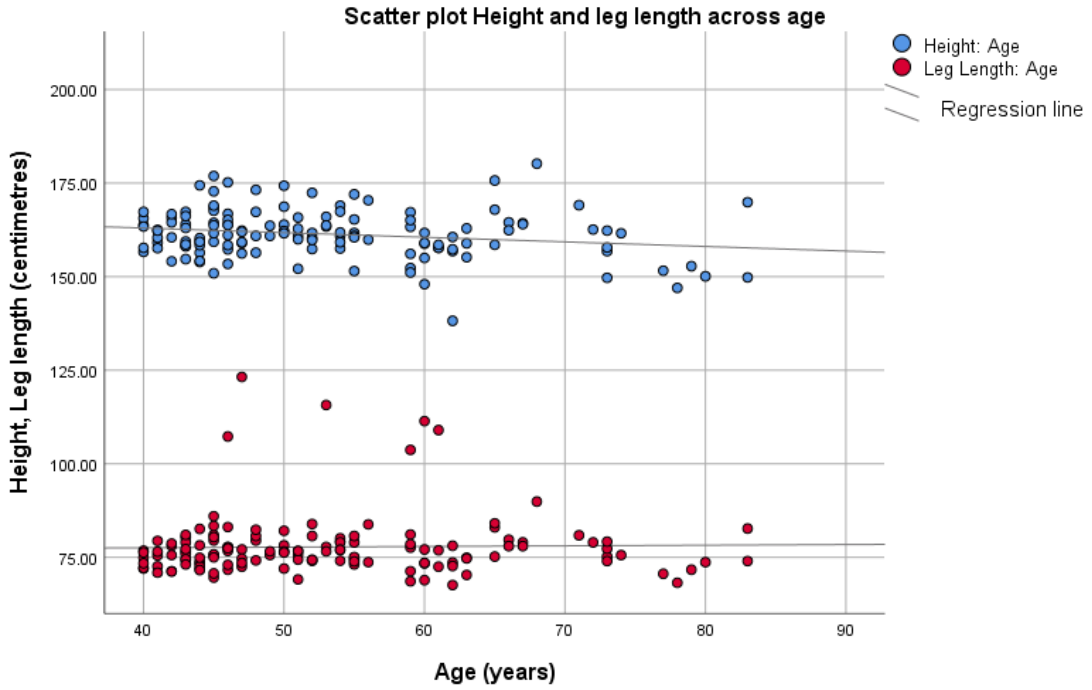
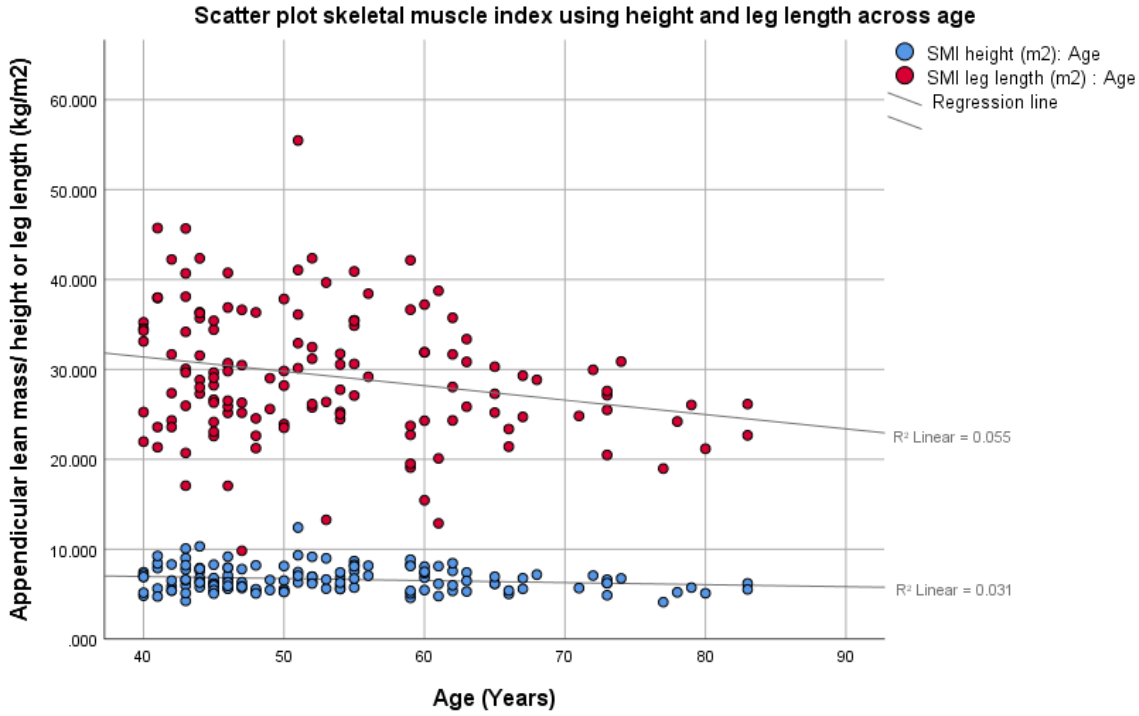


Figure 2.5.5b: Scatter plot showing skeletal muscle mass index using height or leg length across age



2.6 Discussion

This study analysed DEXA and anthropometric data obtained from women who were referred to a tertiary centre. Women in this cohort were categorised into two groups by their age, and a comparison of body composition and anthropometry variables was performed between groups. Consistent with other studies [334, 335], younger women have higher skeletal muscle mass and bone mineral density. Despite the age difference, the prevalence of low skeletal muscle mass by DEXA criteria was similar between both groups. As the majority of the group who were referred had a medical condition (76%, Table 2.5.1a), this possibly reflects a higher sarcopenia risk in those with medical comorbidities.

Relationship between lean and fat mass to bone:

Although lean mass is positively associated with bone, the relationship between fat mass and bone continues to be debated. Conflicting reports have shown both positive [336, 337] and negative [296, 297, 338] relationships. Moreover, this relationship is considered stronger in women than men [298, 321].

Our analysis demonstrates a positive relationship between lean and fat mass to bone mineral content and bone mineral density. Fat mass maintained a strong relationship with bone even after adjusting for skeletal muscle mass and age. Furthermore analysis in a subgroup of women older than 80 years indicates a stronger relationship between fat mass to bone, over lean mass. There are two plausible explanations for this.

Cholesterol is a precursor to oestrogen production in the ovary [339] which plays a key role in bone metabolism [340]. In the post-menopausal state, the oldest of these women are at a higher risk of osteoporosis due to the lack of oestrogen production from the ovary. Therefore older women may be more dependent on fat mass for oestrogen production through aromatization of androgens within fat. A second explanation is the dependence on fat mass as a mechanical load for maintenance of bone strength. Skeletal muscle loss occurs

with increasing age with a higher rate of decline in older individuals, resulting in unintentional weight loss and less mechanical load on the bone. Hence by increasing fat mass resulting in increased mechanical load, bone strength is preserved.

Increase fat mass index have also been shown to be detrimental to bone. In the Busselton healthy aging study by Zhu [300] an increase in FMI > 20kg/m^2 was associated with a decline in bone mineral density. To examine this in our group of women, those with FMI > 20kg/m^2 were selected for a subgroup analysis. Our results showed a persistent positive association between fat mass to bone mineral density, but a stronger relationship between appendicular lean mass to bone when fat mass was corrected for height. To contrast this with Zhu's study, their participants were younger (age 45-67 years) and were recruited from an electoral role. Their findings are more so representative of the general population, while our findings are more in keeping with a group of women with heterogeneous medical conditions relevant to the local population. Given the increased risk of low skeletal muscle mass in those with chronic illness and older women, it is likely that both lean and fat mass have an important role to play in preserving bone strength in our population who are less fit and older than the women in Zhu's study.

The relationship between lean, fat mass and bone are dynamic as myocytes, adipocytes and osteocytes share a common origin in mesenchymal stem cells [293]. Furthermore osteokines released from bone can regulate stem cells in fat and skeletal muscle, and fat mass influences pancreatic beta cell secretion of amylin and insulin which also affects bone [294]. Osteosarcopenia, an emerging entity is recognised to be detrimental to bone due to increased fat infiltration in the muscle and bone [341-343]. Recent studies suggest increased visceral fat is associated with reduced bone density [323, 344] while peripheral fat has a protective role on bone [345].

Therefore although fat mass is positively associated with bone in our study, there may be thresholds at which an increase in fat mass is detrimental. Additionally the distribution of fat either in the peripheral or visceral areas may have differing effects on the bone. These factors are likely influenced by a combination of the interactions between fat, muscle and bone, and hormonal factors such as leptin [346] and insulin. These factors have not been assessed in the current study and would benefit from further exploration in future work.

Relationship between anthropometric variables to skeletal muscle mass on DEXA:

Anthropometry are simple and reliable measurements of limb circumference and skin fold thickness which can be perform at the bedside with a tape measure and handheld skinfold caliper. Mid upper arm [74] and calf circumference [327, 347-349] have been explored as potential screening tools for sarcopenia. In addition, a calf circumference of < 31 cm was associated with impaired physical function and disability in a French study [329]. In the National Health and Nutrition Examination Survey (NHANES) study, Santos showed a regression equation including calf circumference, age and ethnicity correlated well with appendicular skeletal muscle mass on DEXA [327].

In view of the lack of specific anthropometric cut off values using limb circumference for the Australian population, we examined anthropometric measures and its ability to predict low skeletal muscle mass. In our cohort calf circumference was observed to be the strongest predictor of low skeletal muscle mass. Using the EWGSOP 2 criteria, a calf circumference below 35.6 cm provide a sensitivity of 88.6% and specificity of 54.5% in detecting low skeletal muscle mass. These values suggest a calf circumference below this threshold is able to reliably detect, but poorly exclude low skeletal muscle mass. We were unable to compare our data given the lack of Australian data. However, the use of calf circumference as a screening tool for low skeletal muscle mass still has its potential particularly in settings where access to DEXA imaging is a challenge (nursing home residents) or when assessment of grip strength (acute unwell hospital inpatients) may be unreliable. An important future

work is to determine if these cut points correlate with measures of grip strength or clinically meaningful outcomes (disability and mortality).

Exploration of leg length as a denominator in the skeletal muscle mass index:

Leg length when plotted across age, appeared unchanged in older women despite lower height. Considering the use of height in the skeletal muscle index, we examined leg length as a denominator in the skeletal muscle index.

A scatter plot using LL-SMI showed a more obvious decline in older women compared to H-SMI. In the 137 women examined, there was a significant difference in both H-SMI and LL-SMI. This finding suggests leg length may be a useful denominator in the use of skeletal muscle index and warrants further exploration.

Strengths and limitations:

The strengths of this study are that of data analysis which was obtained from a cohort of women from South East Victoria, Australia providing data relevant to the local population. It is the first study to evaluate calf circumference and its threshold value for predicting low skeletal muscle mass in a group of Australian women with heterogeneous medical conditions. Furthermore the study has also shown leg length may be a useful denominator in skeletal muscle index considering it does not change in the older population.

There are however several limitations. Because of the cross sectional nature of the study, findings cannot imply causation. The lack of muscle quality measures and clinical outcomes such as grip strength, impaired function and mortality limits further analysis to determine if the calf circumference cut point and LL-SMI does correlate with clinically meaningful measures. With regard to the relationship between fat mass and bone, there are other

confounders of bone density (smoking history, medications, physical activity, hormonal factors) which were not accounted for which can provide further robustness to the study finding.

Although the study identified calf circumference as a strong predictor of skeletal muscle mass, and the potential use of leg length in the skeletal muscle index it needs to be highlighted that the analysis obtained were from a small cohort of study participants which may not be representative of the general population. An avenue for future research is to explore the utility of calf circumference as a bedside screening tool in a clinical setting (nursing home residents, acute hospital inpatients) where mobility may be impaired and to correlate and compare these findings with meaningful clinical measures of skeletal muscle strength, function and disability scores. Similarly with the use of LL-SMI comparison of this index with H-SMI in a larger cohort and its correlation with measures of muscle function could provide further insights.

2.7 Conclusion

To summarize:

- Skeletal muscle mass and fat mass in this group of Australian women was significantly associated with bone mineral density. This relationship held true across a wide age range and was stronger for skeletal muscle mass than fat mass.

- Calf circumference is a good predictor of low skeletal muscle mass in this cohort although this requires further exploration to determine its correlation with measures of skeletal muscle function.

- Leg length appears unchanged in older women and may be a better denominator than height in the skeletal muscle index in identifying subtle differences in SMI

between groups. As height is lower in the older age group, H-SMI may obscure differences in skeletal muscle index in older cohorts.

Future work would benefit from exploring these findings in a large cohort where its clinical utility and correlation with functional measures and clinical outcomes can be assessed.

Acknowledgements:

1. Professor Boyd Strauss for the use of his dataset and assistance with methods used for this study.

Chapter 3: Circulating hormonal markers related to skeletal muscle regulation in women with hip fracture compared to controls

3.1 Abstract

This chapter examines the key hormones implicated in skeletal muscle regulation in a group of women with hip fracture compared to two groups of women: those awaiting total hip replacement and healthy women from the community. A cross sectional study enrolling forty nine women was performed, comparing the hormonal profile and measures of skeletal muscle mass and grip strength in these women.

The key findings from this study are low skeletal muscle mass and strength in women with hip fracture compared to controls indicating a relatively sarcopenic group of women. Women with hip fracture had lower serum IGF-1, testosterone, IGF binding protein-3 levels, and beta cell function compared to women in the other groups. Linear regression analysis observed a positive relationship between these hormones to measures of skeletal muscle mass. Combined, the study findings suggest these analytes may serve as potential biomarkers for sarcopenia detection in older women with hip fracture particularly in those with mobility impairment which may limit access to skeletal muscle imaging. Furthermore these findings may provide further insight into the mechanism of skeletal muscle loss in these women. Results from this study (focusing on the comparison between hip fracture patients and healthy controls) has been published in the Osteoporosis and Sarcopenia Journal, September 2020, attached in the appendix section.

3.2 Introduction

The link between hormonal systems and skeletal muscle has been discussed in Chapter 1. Serum insulin like growth factor-1 (IGF-1), testosterone [235] and insulin are the main hormones of interest implicated in muscle growth. Increasing age is associated with a

decline in IGF-1 levels, testosterone levels [235] and beta cell function [350], thus potentially contributing to the loss of skeletal muscle with age.

In studies exploring the use of growth hormone and testosterone supplementation in the older population, improvements in muscle mass were reported but evidence around muscle strength and physical performance with hormonal supplementation were modest [351]. In older women, higher free testosterone levels were associated with increased lean body mass [187]. Trials exploring the use of dehydroepiandrosterone (DHEA), a weak androgen, in older women showed mixed results. Some have observed improved muscle function when combined with exercise [195], whilst another study did not detect any improvement in muscle strength [352].

Hip fracture commonly affects older individuals with increased mortality risk in the subsequent year [353]. In those who survive, reduced quality of life and function were reported [354-357]. The association between falls and fracture risk with sarcopenia is further strengthened through a recent systematic review [358]. Moreover the 5 year mortality risk is increased in those with concurrent sarcopenia and osteoporotic hip fracture [359]. Given the increased prevalence of sarcopenia in the hip fracture population [136, 138, 360], a cross sectional study was performed on this group, evaluating the hormonal profile and its association with muscle mass and strength.

Three groups of women were selected for this study: Women with osteoporotic hip fracture, those with osteoarthritic hip awaiting total hip replacement and healthy women from the community. Women with hip fracture were hypothesized to be most sarcopenic, while those awaiting hip replacement would be pre-sarcopenic and healthy controls have lower rates of sarcopenia using the European Working Group on Sarcopenia in Older People 1 (EWGSOP 1) definition [33]. We hypothesized women in the hip fracture group have low

levels of hormones related to skeletal muscle growth compared to women in the hip replacement and healthy control group.

3.3 Aims

- To describe and compare a panel of hormonal biomarkers related to skeletal muscle regulation in women with hip fracture compared to controls

- To assess the relationship between hormonal markers related to skeletal muscle regulation to measures of skeletal muscle mass, strength and sarcopenia status

3.4 Methods

3.4.1 Ethics application, PhD student's role in the study

Under the supervision of Professor Gilfillan, I prepared the study protocol, participant information sheet, data collection sheet and submitted the ethics application. This study was approved by the Eastern Health Ethics Committee approval number HREC/16/EH/104. Participant recruitment, data collection, design of a confidential database with deidentified information was performed by myself. I was also responsible for the collection of fasting blood sample and organisation of skeletal muscle imaging.

Women from this study were consented for collection of muscle biopsy samples for laboratory analysis, reported in Chapters 4 and 5. For women having surgery, I retrieved and processed muscle biopsy samples during their hip surgery. In women from the healthy control group, this was performed under local anaesthesia with assistance received from Professor Gilfillan, Vanessa James and Vanessa Viola, endocrine nurse. Full details are reported in Chapters 4 and 5.

3.4.2 Participant recruitment and selection

All women above the age of 60 were included in the study. Exclusion criteria were pathological fracture, pre-existing neuromuscular condition, steroid use, nursing home resident, those who were unable to consent and those who do not have access to Medicare benefits. At the commencement of the study, it was intended that the groups were age matched. However due to the need for a muscle biopsy, interest from older healthy study participants was lacking, resulting in an older cohort of women recruited from the hip fracture group.

Group 1: Women with hip fracture

Orthopaedic units from Box Hill and Maroondah Hospitals were contacted on a regular basis to obtain a list of women admitted with an acute hip fracture. Women from this group were approached, consented, and enrolled by myself prior to their surgical procedure.

Group 2: Women with osteoarthritis awaiting total hip replacement

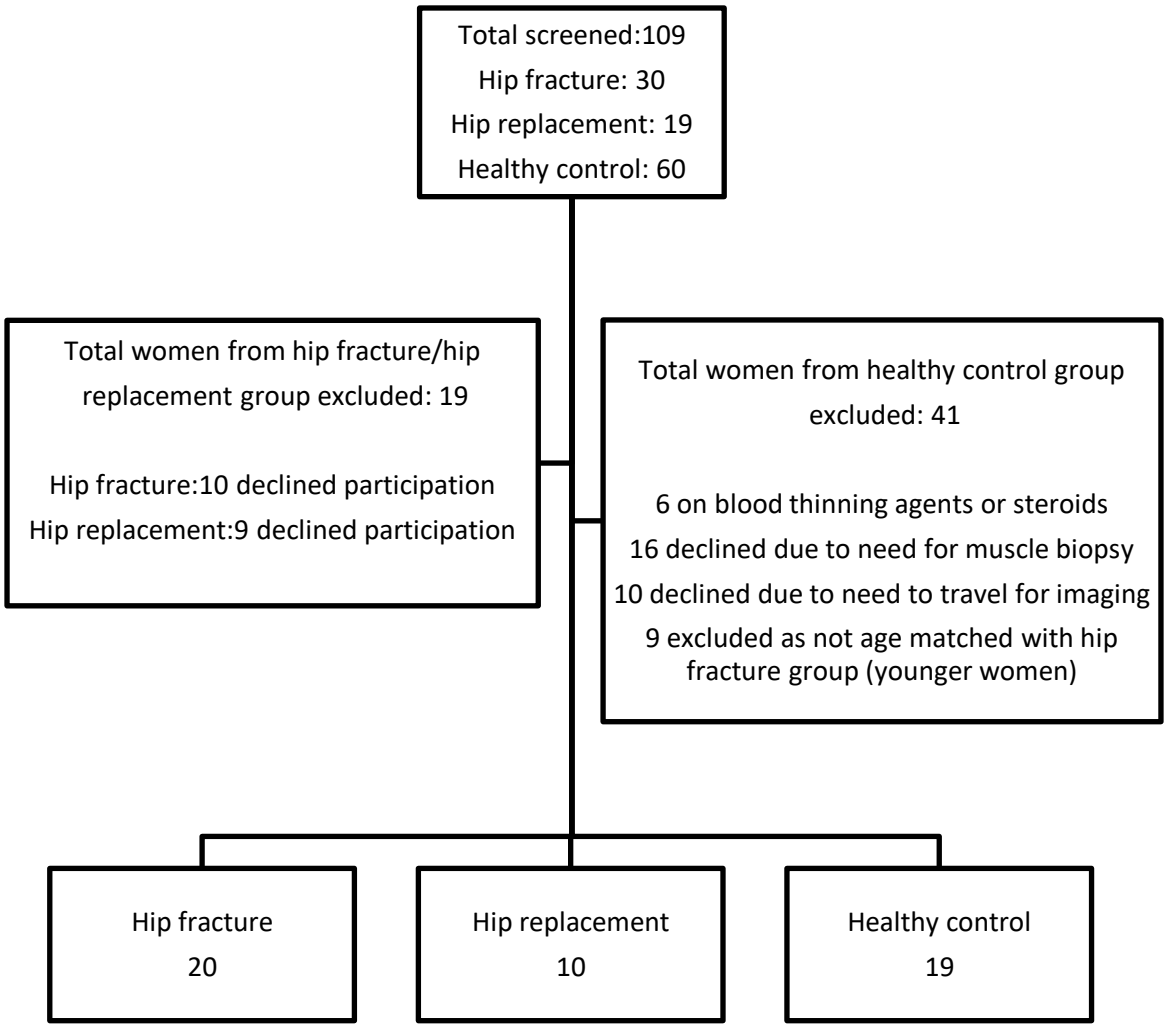
A list of women awaiting total hip replacement for osteoarthritis was obtained from the orthopaedic surgical booking list and screened. Prospective study participants were contacted by myself prior to their scheduled hip surgery to obtain consent and enrolment into the study.

Group 3: Healthy participants from the community

Women from this group were defined as those without previous hip fracture, pre-existing neuromuscular conditions, long term steroid, anticoagulant or blood thinning medication use. Advertisements for the study were placed around hospital notice boards and the local Returned and Services League Club. As the study required a muscle biopsy specimen for laboratory analysis, interest was lacking. Other methods of recruitment for healthy women included placing an advertisement in the local newspaper, and the assistance of Professor

Peteris Darzins who spoke on sarcopenia at a local radio network. There was no remuneration provided, but a paid breakfast meal was offered to this group following their initial visit for fasting peripheral blood sampling. Figure 3.4.2a is a flow chart outlining participant recruitment and selection.

Figure 3.4.2a Flow chart for participant recruitment (Cohort 2)



3.4.3 Bloods sample collection and analysis

Peripheral blood sample collection:

Fasting peripheral blood sample was obtained from all women in the study. Women with hip fracture had their samples collected between Day 1-5 post-operatively. In women from the hip replacement and healthy control group, blood sample were collected before their planned hip surgery or muscle biopsy with an 8 hour fast the night prior.

Peripheral blood sample analysis:

All peripheral blood samples were sent to Eastern Health pathology services for biochemistry and hormonal analysis, with the exception of serum testosterone and insulin like growth factor binding protein-3. These were analysed at Monash Health and Royal Children's Hospital Victoria. Although not specifically a hormone, measurement of insulin like growth factor binding protein-3 in this study was intended to provide a measure of unbound serum IGF-1. Fasting glucose and insulin levels were used to assess beta cell function, calculated with the Homeostasis Model Assessment (HOMA)-Beta equation [361, 362] , and reported as a percentage.

An additional 20 millilitre (ml) of peripheral blood were processed at the Victorian Cancer Biobank located at the Eastern Health Clinical School. Samples were spun for collection and storage of serum and buffy coat for future analysis. This was stored in a -80° C freezer. Details for analytes and their coefficient of variation are provided in Table 3.4.3a.

Serum testosterone levels in this study processed at Monash Health were measured by liquid chromatography mass spectrometry (LCMS). This was performed using Ab Sciex Triple Quad 5500 LCMS system. The analytical range was 0.1-170 nmol/L. Coefficient of variation (CV) was 14.2% for 0.07 nmol/L, 7.8% for 0.16 nmol/L, and 2.7% for 13 nmol/L.

Table 3.4.3a Manufacturer and detection range for biochemistry and hormonal analytes

Analyte (units)	**Manufacturer**	**Measuring range**	**Coefficient of variation (CV)** **Intra assay**	**CV** **Inter assay**
Calcium	Cobas	0.2-5.0 mmol/L	0.5-2.0%	0.8-2.5%
Albumin	Cobas	2-100 g/L	0.4-0.8%	0.5-1.3%
Parathyroid hormone	Cobas	0.127-530 pmol/L	1.1-2.7%	1.1-6.5%
Vitamin D	Cobas	7.5-250 nmol/L	1.6-7.4%	2.6-8.7%
Thyroid stimulating hormone	Cobas	0.005-100 µIU/mL	1.2-8.6%	1.8-8.7%
Free T4	Roche	0.5-100 pmol/L	1.1-5.7%	2.6-10.7%
Free T3	Cobas	0.4-50 pmol/L	1.1-6.5%	1.9-8.2%
Growth Hormone	Cobas	0.03-50 ng/mL	1.3-2.3%	2.7-3%
Luteinizing Hormone	Cobas	0.10-200 mIU/L	0.03-1.41%	1.9-5.2%
Follicle Stimulating Hormone	Cobas	0100-200 mIU/L	1.4-2.8%	2.9-5.3%
Glucose	Cobas	0.11-41.6 mmol/L	0.5-0.7%	1.1-1.3%
Insulin	Cobas	0.2-1000 µU/mL	0.8-3.2%	2.4-4.9%
Serum insulin like Growth Factor-1	Diasorin	100-1500 ng/mL	2.37-4.4%	3.8-8.5%
Serum Insulin like Growth Factor Binding Protein 3	Immuno Diagnostics System	80-10000ng/mL	1.4-2.2%	5.8-7.2%

3.4.4 Measures of muscle mass and strength

To assess for the presence of low skeletal muscle mass, mid-thigh computed tomography (CT), bioelectrical impedance analysis (BIA) and dual energy x-ray absorptiometry (DEXA) was performed. Handgrip strength was used as a measure of skeletal muscle function.

Muscle area by computed tomography (CT):

CT is a gold standard tool for skeletal muscle imaging as it enables differentiation of fat and muscle [57, 363]. In this study, mid-thigh CT was used to evaluate muscle cross sectional area and intramuscular fat infiltration by mean Hounsfield Units (Aquillon Prime, Toshiba, Japan). Mid-thigh area was selected as the imaging site as it correlated well with total body muscle volume [364].

Four slices of 10 mm images were obtained at the level of the mid femur between the symphysis pubis and patella (Figure 3.4.4a). The regions of interest were mapped using Vitrea Core Software. Muscle area was obtained by subtracting the total bone area from the combined total muscle and bone area in mm^2 (Figure 3.4.4b), (outer margins). Mean Hounsfield units was assessed at the region of interest excluding the bone area (Figure 3.4.4c). Normal density muscle was identified by an attenuation of +30 to +100 Hounsfield units (HU) while fat infiltrated low density muscle is defined by attenuation between 0 to +30 HU [57, 365]. Analysis of CT images was performed by me supervised by Stan Tsui and Bjorn Irani, Chief Radiographers at Eastern Health.

Women with hip fracture had their CT imaging performed between Day 1-2 post-surgery. Most women in the hip replacement group had their CT pre surgery except for 4 individuals who were recruited close to their surgery. For these women, CT was performed between Day 1-2 post-surgery. Women from the healthy control group had their CT prior to their scheduled muscle biopsy.

Due to the presence of significant muscle oedema in those who had their imaging performed post operatively, muscle area from the non-operated leg was used for analysis of muscle area. In those who did not have surgery, an average of total muscle area from both legs was selected. As metalware can affect HU reading, data for this measure were obtained from the non-operated leg or from the right leg in those who do not have metalware in situ.

Muscle area was assessed as a continuous variable. The presence of ‘fat infiltrated’ muscle and ‘normal lean’ muscle was defined at the threshold HU of above and below +30 HU, analysed as a dichotomous variable.

Figure 3.4.4a Acquisition of CT imaging at the mid femur level

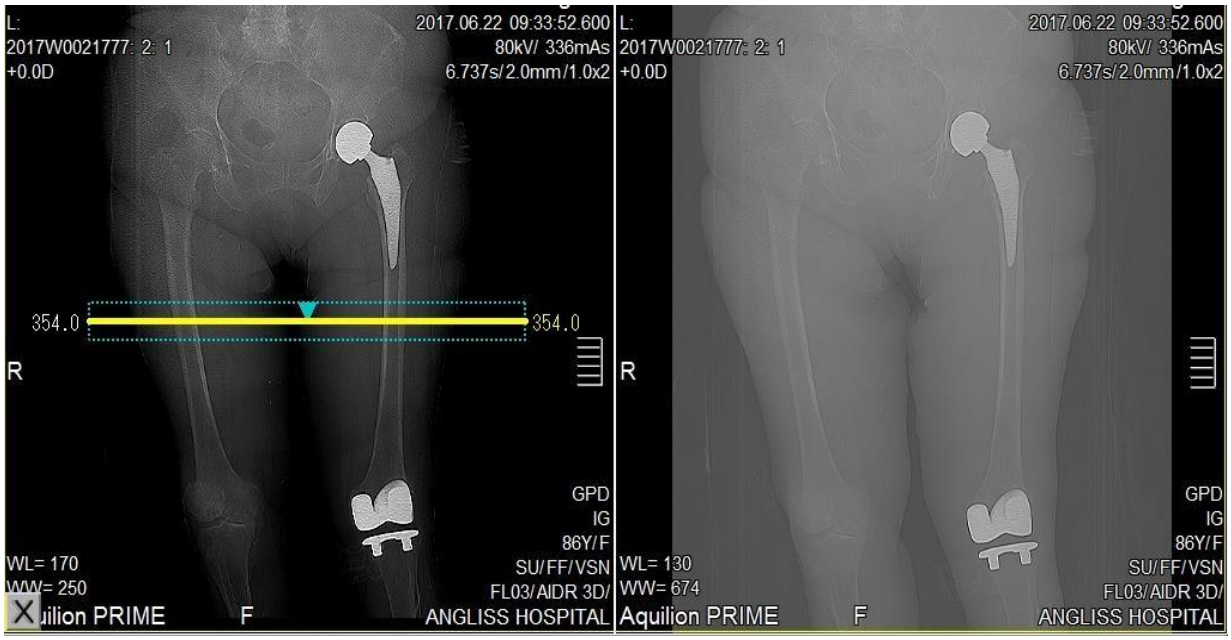


Figure 3.4.4b Estimation of mid-thigh muscle area

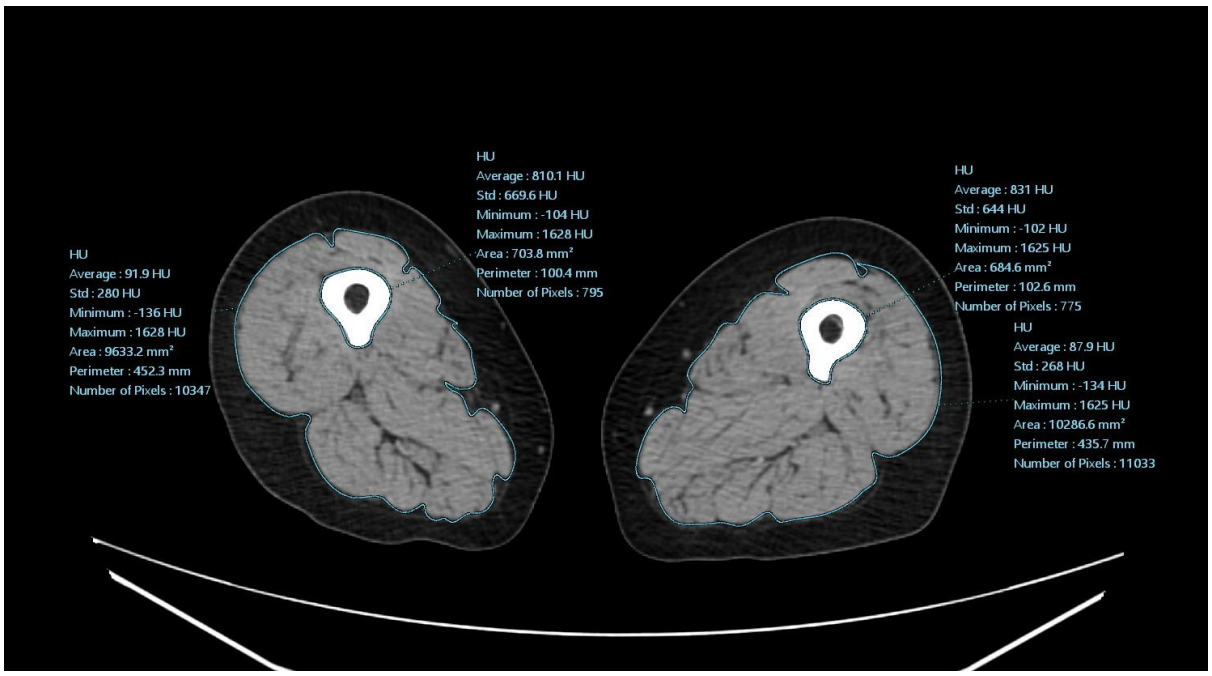
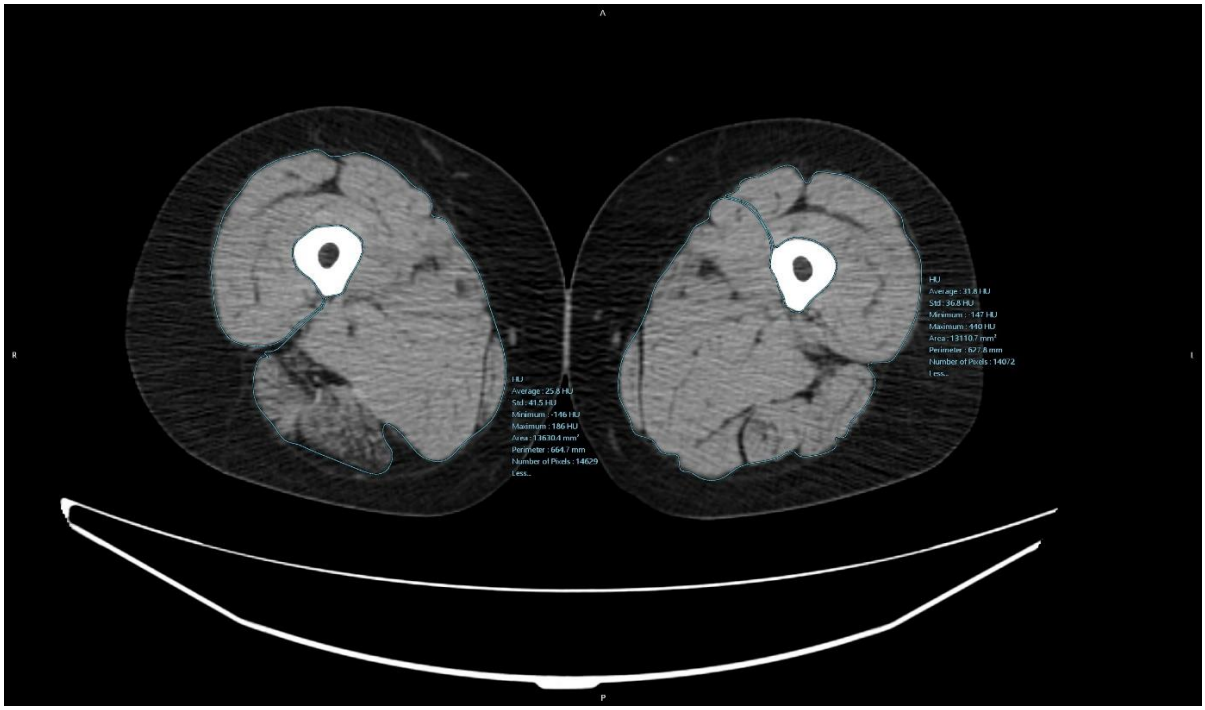


Figure 3.4.4c Estimation of mid-thigh Hounsfield units



Skeletal muscle mass by bioelectrical impedance analysis (BIA):

All women had BIA assessment with the Bodystat MDD 1500 machine (Isle of Man). The test is carried out with the participants lying supine. A pair of electrodes are placed on the anterior aspect on the forearm behind the knuckles and wrist (at the ulnar head) and another pair of electrodes on the anterior aspect of the foot behind the 2nd toe (at the metatarsal area) and mid ankle (between the lateral and medial malleoli).

BIA assessment in the hip fracture group was performed between Day 1-2 post-operatively. In women awaiting hip replacement, their BIA was measured prior to scheduled surgery and women from the healthy control group had their BIA measurement performed prior to muscle biopsy.

The BIA machine provided data on lean mass, dry lean mass, fat mass and resistance data at 50 kilohertz. Absolute skeletal muscle mass was calculated using the regression equation by Janssen [93] utilising resistance data with the following formula:

$$\text{Skeletal muscle mass (kg)} = [(\text{height}^2)/\text{BIA resistance} \times 0.401) + (\text{gender} \times 3.825) + (\text{age} \times -0.071)] + 5.102.$$

Height (cm), BIA resistance in ohms, age (years), gender: men (1), women (0), age (years)

Cut points for skeletal muscle mass index (SMI) by Janssen below 6.75 kg/m^2 and 5.75 kg/m^2 in older women corresponded to increased odds of developing physical disability by 1.41 and 3.31 respectively [75]. Hence for this study, we selected a cut point SMI of <6.75 kg/m^2 to define low skeletal muscle mass by BIA.

Skeletal muscle mass by Dual Energy X-Ray Absorptiometry (DEXA):

Skeletal muscle mass was also measured by DEXA (Hologic Discovery). DEXA imaging was performed as an outpatient either post hospital stay (in the hip fracture group) or prior to surgery (in the hip replacement group) due to limited access to the machine. Calibration of the machine was performed daily with a phantom. The coefficients of variation are 1.2%, 1.4% and 1.9% for lumbar spine, total femur and femoral neck.

There was significant drop out rate in women from the hip fracture group as some of the participants either declined to return for their DEXA or were unable to lie flat for the procedure (including 1 participant from the hip replacement group). Final DEXA data was available from 12/20 women in the hip fracture group, 9/10 from the hip replacement group, and 19/19 from healthy controls. In this study, the EWGSOP1 DEXA SMI cut point < 5.55 kg/m^2 was used to define low skeletal muscle mass by DEXA [33].

Skeletal muscle function by grip strength:

Grip strength as a measure of muscle function was performed on all women using a hand-held Jamar Dynamometer. Readings from 3 attempts were obtained (2 from the dominant arm, 1 from non-dominant arm), and an average was taken as the final grip strength. Gait speed was not assessed due to mobility limitations in the hip fracture group.

Frailty assessment:

Frailty status was assessed using the Clinical Frailty Scale (CFS) [366, 367]. This scale ranges from 1 (Very Fit) to 9 (Terminally Ill). The use of CFS in hip fracture patients has been shown to associate with post-operative complications, length of stay and discharge destination [368].

Sarcopenia status definition:

Sarcopenia in this study was defined by the presence of low skeletal muscle mass and muscle grip strength. We elected to use the EWGSOP 1 [33] definition as all participants were Caucasian. Due to the lack of DEXA data in this study, sarcopenic women were defined by BIA SMI < 6.75 kg/m^2 and grip strength < 20kg according to the EWGSOP1 criteria. Since commencement of this project, the EWGSOP 1 criteria were updated to EWGSOP2 [34]. For completeness prevalence of sarcopenia using these criteria were also reported, with the use of low grip strength adjusted to < 16kg for the EWGSOP2 criteria.

3.4.4 Sample size calculation and statistical analysis

Estimation of sample size was performed in reference to a study by Malkov [369]. To detect a clinically significant 10% difference in skeletal muscle cross sectional area between groups, with a calculated power of 80% and 5% alpha, an estimated minimum of 31 women were required for the study.

Parametric data were expressed as means ± standard deviation. Non-parametric data were reported as median [interquartile range (IQR)]. Student's T-test or Mann Whitney U tests were used to compare two different groups, with ANOVA and Kruskall Wallis for comparison of means and medians between all three groups. Chi square analysis was used to compare sarcopenia status (yes/no) and intramuscular adipose tissue by HU below +30 HU (yes/no) between groups. Analyses were performed using SPSS statistical software version 26 (IBM). p value of < 0.05 was considered statistically significant.

The relationships between biochemistry and hormonal variables to muscle mass and strength were analysed using simple linear regression analysis. As the groups differed by age, multiple regression analysis was performed adjusted for age as a confounder. Binary logistic regression analysis was used to assess the association between biochemistry and hormonal variables to sarcopenia status (yes/no) by EWGSOP 1 criteria. The EWGSOP 2 criteria was not used for the binary logistic regression analysis in this study as there remains a discordance between the new and old criteria EWGSOP criteria [111].

3.5 Results

3.5.1 Demographics

Demographics for the group are reported in Table 3.5.1a. Mean age in the whole group was 76.7 ± 9.8 years old (range 61-99). Women with hip fracture were older than those awaiting hip replacement surgery (p=0.013) but not healthy controls (p=0.073). Women with hip fracture and those awaiting total hip replacement were frailer (p <0.001) and were on more medications compared to healthy women from the community (p <0.002).

Table 3.5.1a: Demographics of study participants

Category	**Hip fracture n=20**	**Hip replacement n=10**	**Healthy controls n=19**	**p value**
Age (years)	80.7 ±11.3 [a]	72.3±6.0 [a]	74.8±8.4	0.05
Weight (kilograms)	67.7±14.1 [a]	83.2± 19.3 [a]	67.6±14.5	0.02
Body Mass Index (kg/m^2)	25.6 (23.6, 31.1)	28 (26.1,32.6)	25.6(23.5,29.0)	0.12
Medication amount	7 (3,10) [b]	7 (4,9)	2 (1,4) [b]	<0.01
Clinical frailty scale score*	3 (2, 5) [b]	3 (2, 3)	2 (1, 2) [b]	< 0.01

* Clinical frailty scale score: 1-Very fit, 2-Well, 3-Managing well, 4-Vulnerable, 5-Mildly frail, 6-Moderately frail, 7-Severely frail, 8-Very Severely frail, 9-Terminally ill.

[a] p value < 0.05 hip fracture compared to hip replacement

[b] p value < 0.05 hip fracture compared to healthy controls

3.5.2 Measures of skeletal muscle mass and strength

Groupwise comparison of measures of skeletal muscle mass and grip strength are reported in Table 3.5.2a. Muscle cross sectional area ($p<0.01$) and grip strength ($p<0.01$) were lowest in women in the hip fracture group, indicating increased risk of sarcopenia in this group. There were trends to lower skeletal muscle mass by DEXA and BIA but these comparisons failed to reach significance.

Women in the hip fracture group had the lowest mean Hounsfield units (HU) compared to women in the other groups ($p<0.01$). Up to 70% of women in the hip fracture group were observed to have increased intramuscular adipose tissue. A higher proportion of women in the hip fracture group were sarcopenic based on both EWSGOP criteria.

Table 3.5.2a Skeletal muscle mass, grip strength and sarcopenia prevalence comparison between groups

Category	**Hip fracture n=20**	**Hip replacement n=10**	**Healthy controls n=19**	**p value**
CT muscle cross sectional area (cm^2)	82.8 ± 16.6 [a b]	101.0 ±17.6 [a]	100± 19.1 [b]	<0.01
CT mean Hounsfield units (HU)	22.0 ± 8.9 [b]	26.4±10.7 [c]	36.2±6.9 [b c]	<0.01
Intramuscular adipose tissue (yes/no) [α]	14/20 (70%)	5/10 (50%)	3/19 (16%)	<0.01
DEXA skeletal muscle mass (kg)	n=13 36.9±4.8	n=9 40.7±7.3	n=19 38.2±6.5	0.37
BIA skeletal muscle mass by Janssen regression equation (kg)	16.6±3.5	19.3±2.7	17.7±3.7	0.14
BIA dry Lean Mass by BIA (kg)	5.8 ± 3.6	8.1 ±2.5	6.9 ± 2.5	0.15
Grip strength (kg)	14.0 ± 6.8 [c]	19.4 ± 7.1	22.9 ± 5.9 [c]	< 0.01
Sarcopenia by EWGSOP1 †	12/20 (60%)	2/10 (20%)	4/19 (21%)	0.02
Sarcopenia by EWGSOP 2 ®	10/20 (50%)	2/10 (20%)	1/19 (5%)	<0.01

† Sarcopenia defined as low skeletal muscle mass by BIA (skeletal muscle mass index < 6.75kg/m^2) combined with low grip strength (<20kg)

® Sarcopenia defined as low skeletal muscle mass by BIA (skeletal muscle mass index < 6.75kg/m^2) combined with low grip strength (<16kg)

[α] Mean HU < 30 HU (yes/no)

[a] p<0.05 Hip fracture group compared to hip replacement

[b] p< 0.05 Hip fracture group compared to healthy controls

[c] p<0.05 Hip replacement compared to healthy controls

3.5.3 Biochemistry, hormonal profile and beta cell function.

No significant difference was detected in corrected calcium, parathyroid hormone and vitamin D levels between groups. Albumin levels ($p < 0.01$), free T3 ($p < 0.01$), and free testosterone levels by mass spectrometry were significantly lower in the hip fracture group ($p<0.01$). Women with hip fracture had comparatively lower serum IGF-1(IGF-1) ($p < 0.01$) and IGF Binding Protein 3 levels (IGFBP-3) ($p < 0.01$) compared to healthy controls (Table 3.5.3a).

Table 3.5.3a: Comparison of biomarkers (biochemistry and hormonal levels) in between groups

Category	**Hip fracture (n=20)**	**Hip replacement (n=10)**	**Healthy controls (n=19)**	**p value**
Corrected calcium (mmol/L)	2.4 (2.3, 2.6)	2.4 (2.3, 2.4)	2.4 (2.4, 2.5)	0.66
Albumin (g/l)	30.0 (26.5, 34.0) [ab]	39.0 (37.0, 40.0) [a]	40 (38.5, 41.0) [b]	<0.01
Parathyroid Hormone (pmol/L)	5.0 (4.1, 7.6)	4.2 (3.7, 5.2)	6 (4.8, 7.6)	0.05
Vitamin D (nmol/L)	58.7 ± 33.6	67.2 ± 21.6	67.0 ± 23.1	0.59
Thyroid stimulating hormone (mU/L)	1.8 (0.7, 3.2)	1.2 (1.0, 1.7)	1.7 (1.3, 3.1)	0.48
Free T4 (pmoL/l)	18.1 ± 3.7	16.6 ± 3.0	16.6 ± 2.8	0.29
Free T3 (pmol/L)	3.6 ± 0.7 [b]	4.5 ± 0.9	4.9 ± 0.7 [b]	<0.01
Free testosterone (nmol/L)	4.6 (3.8, 7.0) [b]	5.6 (4.7, 11.0)	9.1 (6.1, 11.8) [b]	<0.01
Insulin like Growth Factor (nmol/L)	11.1± 4.1 [b]	16.2 ±5.8	18.8 ± 5.5 [b]	<0.01
Growth Hormone (ng/L)	0.6 (0.4, 1.8) [b]	1.5 (0.6, 2.6)	1.9 (1.1, 3.0) [b]	0.05
Insulin like Growth Factor Binding Protein 3 (nmol/L)	73.6 ± 28.1[ab]	117.2 ± 31.5 [a]	121.4 ± 28.6 [b]	<0.01
IGF/IGFBP3 ratio	0.2 ± 0.11	0.1 ± 0.03	0.2 ± 0.03	0.45

[a] p <0.05 hip fracture compared to hip replacement

[b] p <0.05 hip fracture compared to healthy controls

To evaluate beta cell function, fasting glucose and insulin was analysed (Table 3.5.3b). Beta cell function was assessed using the HOMA beta calculator [361, 362]. Women in the hip fracture group were observed to have impaired beta cell function compared to the other groups. After exclusion of the individual who was on an insulin secretagogue from the analysis, reduced beta cell function was maintained in the hip fracture group.

Table 3.5.3b Comparison of fasting glucose, insulin and beta cell function between groups

Category	**Hip fracture (n=19) †**	**Hip replacement (n=10)**	**Healthy controls (n=19)**	**p value**
Fasting glucose (mmol/L)	5.7 (2.4,6.5)	5.4 (5.3,5.7)	5.4 (5.2,5.6)	0.15
Fasting insulin (mu/L)	8 (5.5, 12.5)	10 (8, 11)	9 (7, 12)	0.72
HOMA Beta (%)	70.6 (62.4, 80.7) [ab]	93.5 (84.7, 108.0) [a]	90.3 (73.4, 109.3) [b]	<0.01

† Individual on insulin secretagogue excluded from analysis

[a] p value < 0.05 hip fracture compared to hip replacement

[b] p value < 0.05 hip fracture compared to healthy controls

3.5.4 Linear regression analysis between hormonal variables to measures of skeletal muscle mass and strength

Simple linear regression analysis was performed to assess the relationship between variables which were significantly different between groups to muscle mass (muscle area on CT) and grip strength as continuous variables. Serum albumin, IGF-1, IGFBP-3 levels, HOMA Beta and free testosterone levels were positively associated with skeletal muscle mass by CT, independent of age. When assessed with grip strength as the outcome variable, there was a positive association between serum albumin, IGF-1 and HOMA beta to grip strength in the age adjusted model (Table 3.5.4a).

Table 3.5.4a: Linear regression analysis with muscle area on CT and grip strength as dependant variable

Age adjusted model (Muscle area on CT as dependent variable)				
Independent variable	**Dependant variable**	**Beta (95% CI)**	**r^2**	**p value**
Serum albumin (g/l)	Muscle area on CT (cm^2)	1.10 (0.12,2.07)	0.18	0.03
Serum IGF-1 (nmol/L)		1.30 (0.40,2.20)	0.23	<0.01
IGFBP-3 (nmol/L)		0.23 (0.06,0.40)	0.27	<0.01
HOMA Beta (%)		0.48 (0.30,0.65)	0.46	<0.01
Free testosterone (nmol/L)		1.78 (0.09,3.47)	0.22	0.04
Age adjusted model (Grip strength as dependant variable)				
Independent variable	**Dependant variable**	**Beta (95% CI)**	**r^2**	**p value**
Serum albumin (g/l)	Grip Strength (kg)	0.50 (0.24,0.75)	0.61	<0.01
Serum IGF-1 (nmol/L)		0.32 (0.06,0.59)	0.54	0.02
IGFBP-3 (nmol/L)		0.05 (-0.002,0.098)	0.58	0.06
HOMA Beta (%)		0.08 (0.02, 0.14)	0.54	0.02
Free testosterone (nmol/L)		0.28 (-0.23, 0.80)	0.55	0.28

3.5.5 Muscle Hounsfield units to grip strength and hormonal variables

In view of the significant difference between groups in muscle Hounsfield units (HU), the relationship between muscle HU to grip strength was assessed both as a continuous and dichotomised variable. In the dichotomised variable, those with muscle HU < +30 HU were given a score of 1 indicating increased intramuscular fat infiltration. Increased muscle HU was associated with increased grip strength, while those with muscle HU below 30 HU have lowered grip strength. This relationship however was non-significant when corrected for age (Table 3.5.5a)

Table 3.5.5a Linear regression analysis muscle HU to grip strength

Variable	**Beta (95% CI)**	**r^2**	**p value**
Muscle HU (Continuous variable)	0.313 (0.12, 0.51)	0.19	<0.01
Muscle < +30 HU (Yes/No)	-5.623 (-9.81, -1.43)	0.137	0.01
Age adjusted model			
Muscle HU (Continuous variable)	0.122 (-0.05, 0.30)	0.50	0.15
Muscle < +30 HU (Yes/No)	-1.096 (-0.71, -0.33)	0.484	0.56

The relationships between hormonal variables which were significantly different between groups to muscle HU were also analysed in a simple linear regression analysis (Table 3.5.5b). Serum IGF-1 and IGFBP3 were positively associated with muscle HU ($p<0.05$), suggesting increased IGF-1 and IGFBP-3 levels were associated with lower levels of intramuscular fat infiltration.

Table 3.5.5b Linear regression analysis hormonal variables to muscle HU as outcome variable.

Variable	**Beta (95% CI)**	**r^2**	**p value**
Serum IGF-1 (nmol/L)	0.526 (0.03,1.02)	0.091	0.04
IGFBP-3 (nmol/L)	0.098 (0.01,0.19)	0.108	0.03
HOMA Beta (%)	0.078 (-0.05,0.20)	0.034	0.21
Free testosterone (nmol/L)	0.809 (-0.18,1.80)	0.062	0.11

3.5.6 Binary logistic regression analysis between hormonal variables to sarcopenia status.
Binary logistic regression analysis was performed to assess the relationship between hormonal variables to sarcopenia status (yes/no) by EWGSOP1 criteria (SMI by BIA <6.75kg/m^2 and grip strength <20kg).

Hormonal variables to sarcopenia status

In a binary logistic regression analysis, insulin was the only hormonal marker which was significantly associated with sarcopenia status. The results are reported as the odds for sarcopenia for every one unit reduction in the independent variable. For every 1mu/L

decrease in insulin levels, the odds of developing sarcopenia increases by a factor of 1.53. (OR 1.53, p value 0.02) (Table 3.5.6a).

Table 3.5.6a Binary logistic regression analysis with hormonal variables to sarcopenia status by EWGSOP1, adjusted for age

Variable (Age adjusted)	**r^2**	**OR**	**p value (95%CI)**
Serum albumin (g/l)	0.40	1.14	0.07 (0.99, 1.32)
Serum IGF-1 (nmol/L)	0.37	1.09	0.24 (0.94, 1.27)
IGFBP-3 (nmol/L)	0.46	1.02	0.10 (1.00,1.06)
HOMA Beta (%)	0.37	1.02	0.26 (0.99, 1.05)
Free testosterone (nmol/L)	0.43	1.08	0.59 (0.81,1.45)
Fasting insulin (mu/L)	0.49	1.53	0.02 (1.08, 2.17)

3.5.7 ROC curve assessing the ability of a scoring system combining IGF-1, Free testosterone and HOMA Beta to predict low muscle area by CT

As IGF-1, Free testosterone and HOMA Beta were significantly different between groups, these three variables were combined and assessed in a multiple regression analysis to determine its relationship to muscle area by CT and grip strength. Serum IGFBP-3 was excluded from the analysis due to its collinearity with serum IGF-1 (r=0.80). Serum IGF-1, Free testosterone and HOMA Beta had a stronger relationship to muscle area on CT (r^2 =0.535) compared to grip strength (r^2=0.335) (Table 3.5.7a).

Table 3.5.7a Multiple linear regression analysis combining IGF-1, Free testosterone and HOMA Beta to muscle area on CT (mm^2) and grip strength (kg) as dependent variable.

Dependent Variable (Muscle area on CT)	**r^2**	**Beta (95%CI)**	**p value**
Serum IGF-1 (nmol/L)	0.535	69.00 (-5.20,143.20)	0.07
HOMA Beta (%)		48.40 (28.80, 68.10)	<0.01
Free testosterone (nmol/L)		165.90 (33.60,298.20)	0.02
Dependent Variable (Grip strength)	**r^2**	**Beta (95%CI)**	**p value**
Serum IGF-1 (nmol/L)	0.335	0.46 (0.09, 0.83)	0.02
HOMA Beta (%)		0.09 (-0.01, 0.19)	0.07
Free testosterone (nmol/L)		0.39 (-0.28, 1.05)	0.25

To further explore if a scoring system with these variables can be used to predict low skeletal muscle mass by CT, predicted muscle area for each individual using a regression including serum IGF-1, HOMA Beta and Free testosterone was calculated. The regression equation used to calculate predicted muscle area was:

Predicted skeletal muscle area (mm^2) = 3083.43 + 68.99 (Serum IGF-1) + 48.43 (HOMA Beta) + 165.87 (Free testosterone).

To create a receiver operating curve (ROC), muscle area 1 standard deviation (SD) below the true muscle area of the population mean of women in Cohort 2 were selected as threshold to define 'low' and 'normal' muscle mass. A score of 88.55 cm^2 (incorporating serum IGF-1, HOMA Beta and free testosterone) provided a sensitivity of 87.5% and specificity of 61.1% in predicting true low muscle area by CT. Area under the curve (AUC) was 0.847, p value=0.002 (0.679,1.000) (Figure 3.5.7a).

Figure 3.5.7a ROC curve using muscle area < 1 SD population mean (Cohort 2)

3.6 Discussion

The aim of this study was to characterise and compare hormonal biomarkers related to skeletal muscle regulation in women with hip fracture compared to two groups of controls: women awaiting total hip replacement and healthy controls. Although women in the hip replacement group were likely to be sarcopenic, they were recruited in the anticipation that recruitment of healthy controls would be challenged by planned muscle biopsy. Attempts to age match the participants were unsuccessful resulting in an older cohort of women with hip fracture. In our analysis, as it became apparent women awaiting hip replacement were as sarcopenic as women with hip fracture and were not ideal controls, reporting of data from this group was omitted in the submitted publication.

Pertinent findings from this study were significantly lower albumin, serum IGF-1, IGFBP-3, free testosterone levels and beta cell function in the hip fracture group compared to women from the other groups. These findings are discussed in the subsequent sections. Low albumin levels in the hip fracture cohort are consistent with other studies [370] suggesting impaired nutrition is a risk factor for hip fracture. Alternatively, the loss of albumin from inflammation and leakage into the extravascular space can occur in critically ill patients [371], and could be another reason for this observation in women who have just undergone surgery.

Low levels of serum IGF-1 and IGFBP-3 were detected in the hip fracture group. These variables had a positive relationship to skeletal muscle mass and an inverse relationship to intramuscular fat infiltration (by HU). Although the anabolic effect of IGF-1 on muscle is known, data examining the association between IGFBP-3 and muscle are scarce. Due to its short half-life, the majority of IGF-1 in the circulation are predominantly bound to IGF binding proteins (IGFBP) [207] and acid labile subunit [372]. Of the six IGFBP identified, IGFBP-3 is the most abundant, and thus was examined in our study to provide information on the activity of unbound IGF-1.

Conventionally the IGFBP were considered as carrier proteins [373]. It inhibits IGF-1 signalling by ligand sequestration [372] and binding to IGF-1 receptors. Subsequent evidence reveal IGFBP also enhances IGF-1 signalling in an autocrine/paracrine manner by concentrating IGF-1 around local sites, and promote proteolytic cleavage of bound IGF-1 from the IGF-1/IGFBP complex allowing unbound IGF-1 to interact with its receptors [372].

There are also evidence IGFBP-3 exert its effects independent of the IGF-1 pathway. The IGFBP-3 is involved in the inhibition of cell growth, promotion of apoptosis [374], and cell senescence in aging [373] by up regulation of Forkhead Box O, (FOXO) a pathway involved in

disuse muscle atrophy [375]. Moreover, IGFBP-3 also interacts positively with the TGF-β/SMAD pathway (reviewed in Chapter 1) in smooth muscle and placental tissue [219]. Of the vast members of the TGF-β superfamily, myostatin is well implicated in muscle wasting. Taken together these findings indicate IGFBP-3 may have a role in skeletal muscle atrophy. Conversely, there are also studies where IGFBP-3 has a positive effect on skeletal muscle growth. IFGBP-3 was demonstrated to support myoblast differentiation [376] and upregulation of mTOR [377]. Administration of the IGF-1/IGFBP3 complex to alcohol fed mice has been found to attenuate the adverse effects induced by chronic alcohol ingestion on muscle protein synthesis [378].

Our study findings were more in keeping with a positive association between IGFBP3 on the muscle rather than a detrimental one. This is consistent with findings from other studies in hip fracture [379] and stroke patients [380]. Despite lower levels of IGF-1 and IGFBP-3 in women with hip fracture, IGF-1/IGFBP-3 ratios did not differ between groups. This observation is due to the strong correlation between IGF-1 and IGFBP3 ($r=0.80$) indicating an interdependent relationship between IGF-1 and IGFBP-3 in our women. Although not examined in this study, it is possible IGFBP-3 have diverse roles to play in skeletal muscle regulation through its interaction with both stimulatory (via IGF-1) and atrophy (via TGF-β/SMAD and FOXO) pathways to maintain a balance in net protein turnover. The association between IGFBP-3 and some of these pathways are examined in Chapter 5.

Testosterone levels were lowest in the hip fracture group and were positively associated with measures of muscle area by CT and grip strength in keeping with previous findings using DEXA and bioelectrical impedance analysis [187, 381]. While conventionally considered a male hormone, this study now supports the role of testosterone as a potential muscle regulator with positive effects in women. Following adjustment for age, analysis did not detect a significant relationship between testosterone levels to grip strength. In this

setting, free testosterone levels was a better indicator of low skeletal muscle mass but not reduced function.

Another finding is that of lowered beta cell function by homeostasis model, an indicator of intrinsic insulin production in the hip fracture group. Insulin is secreted by the pancreatic beta cells and works by enhancing glucose uptake via the glucose transporter. In the presence of amino acids, insulin prevents protein breakdown and increases protein synthesis through activation of the mTOR1 signalling [237, 382, 383] in the muscle. In the current study, HOMA beta was positively associated with skeletal muscle area and strength independent of age. This finding combined with the positive relationship between insulin to sarcopenia status further supports the role of insulin in skeletal muscle regulation in these women.

Women in the hip fracture group had the lowest muscle Hounsfield units indicating increased intramuscular adipose tissue in the thigh region. Increased intramuscular fat infiltration is known to affect muscle quality and function [384] and thus may contribute to increased sarcopenia risk in these women. Analysis in this study shows a significant positive relationship between muscle HU and grip strength. This relationship however was non-significant after adjustment for age, suggesting age is a stronger predictor of grip strength and is closely related to increasing fat infiltration in muscle.

The three variables (IGF-1, free testosterone, HOMA Beta) were all combined to create a scoring system to predict skeletal muscle mass. Predicted values were used to assess against true muscle area 1 SD below the population mean as a threshold. A score of 88.55 cm^2 provided a sensitivity of 87.5% and specificity of 61.1% in predicting true low muscle area by CT.

Current sarcopenia guidelines recommend the presence of low muscle function and mass which requires the use of specific tools (BIA/DEXA/CT/handheld dynamometer). In settings where access to these tools is limited, the measurement of these three variables with a simple peripheral blood sample could prove useful as a screening option for sarcopenia detection in older women. This combined with the specific muscle area cut points identified will need further validation in a larger cohort of women.

Strengths and limitations:

The strengths of this study is the characterisation and comparison of hormonal profile in a local sample providing data specific to a local population. The finding of impaired beta cell function in the hip fracture group has not been reported before and provides new knowledge.

However there are several limitations. Although the study was adequately powered to detect a difference in muscle area by CT, the small sample size limits the generalizability of the study findings. While a bigger study population would be ideal, the challenges around participant recruitment within the exclusion/inclusion criteria combined with a need for muscle biopsy and further laboratory analysis to generate substantial knowledge for this thesis were limited by thesis submission deadlines. Regardless findings from this study can still form a basis for the characterisation of hormonal profile for future large scale population studies in older women with hip fracture. Understanding the link between hormonal levels and its interaction with molecular pathways involved in skeletal muscle regulation may provide further insights towards the exploration of potential treatment options.

Peripheral blood samples for patients with hip fracture patients were collected post-operatively. As there were no serial follow up blood samples collected it is uncertain if the

changes in hormonal levels are related to acute illness and/or surgery. However when analysis was performed excluding women with hip fractures, the relationship between IGFBP3 and HOMA beta to muscle area was maintained indicating some of the changes observed are not specifically related to being acutely unwell (data not shown). The lack of DEXA data limits its utility in this study as a measure for low muscle mass and further analysis. It is likely those who returned for their DEXA scans post operatively were less frail and less sarcopenic. This therefore explains the lack of difference observed in skeletal muscle mass between groups by DEXA.

3.7 Conclusion

Women with hip fracture have a high prevalence of sarcopenia. Low serum IGF-1, IGFBP-3, free testosterone, and impaired beta cell function were detected in these women and may serve as potential biomarkers for sarcopenia. Given the link between IGF-1 signalling to mTOR and the association between IGFBP-3 to TGF-β, exploring these pathways would be useful to determine if they have role in muscle wasting in older women with hip fracture. Characterisation of the hormonal profile and the exploration of these four variables in predicting low muscle mass and function in a larger cohort would prove useful to confirm the generalisability of our study findings. By further examining the link between hormonal levels and its interaction with the molecular pathways involved in skeletal muscle regulation, further understanding may lead to the exploration of potential treatment options for ameliorating sarcopenia which is highly prevalent in the hip fracture population.

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3.8 Appendix

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Original article

Sarcopenia in women with hip fracture: A comparison of hormonal biomarkers and their relationship to skeletal muscle mass and function

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ABSTRACT

Objectives: Sarcopenia is a decline in skeletal muscle mass and function. It is associated with adverse outcomes and increased mortality. Sarcopenia is also reported to be prevalent in the hip fracture population. Our aims in this study are to compare the hormonal profile in women with hip fracture to controls, and to assess the relationship between hormonal biomarkers to skeletal muscle mass and function in these women.

Methods: A cross sectional study was performed enrolling women above age 60 years old with hip fracture as a study group. For comparison healthy women from the community were recruited. Peripheral blood samples were obtained for analysis of hormonal profiles. Measures of skeletal muscle mass and function by muscle area on computed tomography, dual energy X-ray absorptiometry, bioelectrical impedance analysis, and grip strength was performed.

Results: A high proportion of sarcopenic individuals were detected in the hip fracture group (60%). Women with hip fracture compared to controls were older ($P = 0.073$), had lower serum albumin levels ($P < 0.001$), serum insulin-like growth factor-1 (IGF-1) ($P < 0.001$), insulin-like growth factor binding protein −3 (IGFBP-3) ($P < 0.001$), free testosterone levels ($P = 0.001$), and impaired beta cell function by homeostasis model assessment (HOMA beta) ($P = 0.038$).

Conclusions: There is a high proportion of sarcopenic individuals in the hip fracture group. Lowered serum levels of IGF-1 and IGFBP-3, HOMA beta cell function, and free testosterone levels were detected in this group and may serve as potential biomarkers of sarcopenia.

1. Introduction

Sarcopenia is a decline in skeletal muscle mass and function. It is more common in the older population but can also be seen in younger individuals with chronic diseases [1–3]. Since its initial description by Rosenberg in 1997 [4], sarcopenia is now listed as a condition in the International Classification of Diseases-10 [5], enabling better recognition and detection. Further research have also shown the association between sarcopenia and the risk of several adverse outcomes. These include prolonged hospital stay [6], increased falls and fracture risk [7,8], in addition to increased morbidity [9] and mortality [10–12]. Despite these findings, there are currently no pharmacological therapies for sarcopenia and this lack of progress likely relates to the complex balance between anabolic and catabolic pathways involved in skeletal muscle regulation [13].

Muscle growth is also affected by hormonal factors such as serum insulin like growth factor-1 (IGF-1) and testosterone [14]. A decline in these hormonal levels with increasing age contributes further to the loss of skeletal muscle mass with aging. In studies

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assessing growth hormone replacement in the older population, improvement in lean body mass but not muscle strength was observed [15], whilst physiological testosterone therapy in hypogonadal men only showed modest improvement in muscle mass and strength [16].

In older women, higher free testosterone levels were also associated with increased lean body mass [17]. Studies evaluating the use of dehydroepiandrosterone (DHEA), a precursor androgen, and its effects on muscle strength in older women have shown mixed results with 1 study reporting improvements in sitting leg strength and physical function assessed by the Short Physical Performance Battery Score [18], while another study did not detect any changes in muscle strength [19].

Hip fracture commonly affects older individuals with increased mortality risk in the subsequent year [20]. In those who survive, reduced quality of life and function have been reported [21–24]. The association between falls and fracture risk with sarcopenia is further strengthened through a recent systematic review [8], and the 5-year mortality risk is increased in those with concurrent sarcopenia and osteoporotic hip fractures [25]. Given the increased prevalence of sarcopenia in the hip fracture population [26–28], we performed a cross sectional study on this group, evaluating the hormonal profiles in these women and its association with skeletal muscle mass and strength.

We also assessed the relationships between hormonal levels to sarcopenia status (sarcopenia/no sarcopenia) by the European Working Group on Sarcopenia in Older People (EWGSOP) 1 criteria [29]. Since commencement of this project the EWGSOP criteria have been updated to EWGSOP2 [30]. Analysis was reported using both criteria.

The aim of this study is to describe and compare a panel of hormonal biomarkers related to skeletal muscle regulation in women with hip fractures, compared to controls. We hypothesized women with hip fractures compared to controls were more likely to be sarcopenic and have altered hormonal biomarker profiles related to skeletal muscle regulation.

2. Methods

Thirty-nine women aged above 60 years old were enrolled into the study between June 2016 and July 2019. Twenty women with hip fractures were recruited during their hospital admission as the study group. For comparison 19 healthy women from the community were recruited by community advertisements. All women also consented to collection of muscle biopsy for analysis of the molecular pathways involved in muscle regulation. These findings will be submitted as a separate paper. All participants provided informed consent prior to inclusion in the study. All procedures performed involving human participants were in accordance with the ethical standards of the Eastern Health Ethics Review Committee and was performed in accordance with the Helsinki Declaration of 1975, Eastern Health HREC reference number HREC/16/EH/104.

We were also interested in collecting data on length of stay on the rehabilitation ward (not reported in this study). As nursing home residents were generally discharged back to residential aged care facilities without a period of rehabilitation thus precluding the ability to collect this data, they were excluded from the study. Women with pre-existing neurological conditions which are known to affect skeletal muscle were also excluded. Women in the healthy control group were excluded if they were on blood thinning agents which would preclude muscle biopsy.

Sample size calculation was performed in reference to a study by Malkov [31] whereby there was 10% significant difference in muscle area reported in between groups. To detect a 10% difference in skeletal muscle cross sectional area between our groups, with a calculated power of 80% and 5% alpha, we estimated that a minimum of 31 women were required for the study. A total of 90 women were screened. Following exclusion, a total of 39 women were enrolled into the study.

Fasting peripheral blood samples were obtained for analysis of biochemistry, fasting glucose, insulin and hormonal levels related to skeletal muscle regulation. In women with hip fractures, fasting peripheral blood sample was collected within 1–5 days post-surgery. Frailty status were recorded using the Clinical Frailty Scale (CFS) [32,33]. This scale ranges from 1 (Very Fit) to 9 (Terminally Ill).

Skeletal muscle mass was assessed using dual energy X-ray absorptiometry (DXA) (Hologic Discovery, New South Wales, Australia), muscle cross sectional area on mid-thigh computed tomography (CT) scan (Aquillon Prime, Toshiba, Japan), and calculation of skeletal muscle index using the Janssen regression equation [34] utilising resistance data from bioelectrical impedance analysis machine (MDD1500, Body Stat Limited, Isle of Man).

Coefficient of variation for the DXA scan as provided by manufacturers are 1.2% lumbar spine, 1.4% total femur, and 1.9% femoral neck. Due to limitations around access to DXA imaging, all DXA was performed on an outpatient basis. DXA data were unavailable in 8 women from the hip fracture group who were either unable to tolerate the procedure or have declined to return for their imaging. In view of the relative lack of DXA data, prevalence of sarcopenia was assessed using data from bioelectrical impedance analysis (BIA).

Mid-thigh CT in the hip fracture group were performed within 1–3 days post-surgery. As there was a significant amount of muscle edema observed on the operated leg, muscle area from the non-operated leg was used for analysis in this study. In healthy controls an average of muscle area in both legs combined were used as a measure of skeletal muscle mass.

Skeletal muscle function by grip strength was measured on a Jamar handheld dynamometer. An average reading of 3 attempts were taken as the final grip strength.

The European Working Group on Sarcopenia in Older People (EWGSOP) 1 and 2 [29,30,34] criteria were used to define sarcopenia as all study subjects were Caucasian. Specific cut-off values used from both criteria to define sarcopenia are as listed:

EWGSOP1: Skeletal muscle mass index by BIA, adjusted for height <6.75 kg/m^2 and grip strength <20 kg.
EWGSOP 2: Skeletal muscle mass index by BIA, adjusted for height <6.75 kg/m^2 and grip strength <16 kg.

We have presented a parallel analysis using both criteria.

Student's T test were used to compare means between groups. Chi square test were used to compare sarcopenia status between groups. Analysis were performed using SPSS statistical software programme version 25 (IBM). A P value of < 0.05 was considered statistically significant.

The relationship between different biochemistry and hormonal variables to muscle mass and function were analyzed using univariate regression analysis followed by multiple regression analysis. As the groups differed by age, multiple regression analysis were performed adjusted for age as a confounder.

Binary logistic regression analysis were performed with the

participants further divided into sarcopenia status (yes/no) assessing the relationship between the different hormonal variables to sarcopenia status.

3. Results

3.1. Groupwise comparison: Participant's characteristics

Mean age in the whole group was 77.82 ± 10.31 years old (range 61–99). Women with hip fractures were older (80.70 ± 11.30 vs 74.79 ± 8.43 years, P = 0.073), frailer (3 ± 2 vs 2 ± 1, P < 0.001) and were on more medications (6 ± 4 vs 3 ± 2, P = 0.001) compared to healthy women from the community (Table 1).

Muscle cross sectional area (82.82 ± 16.61 vs 100.01 ± 19.09 cm^2, P = 0.005) and grip strength (14.01 ± 6.83 vs 22.93 ± 5.94 kg, P < 0.001) were lower in women in the hip fracture group, indicating increased sarcopenia risk in this group. A higher proportion of women in the hip fracture group were sarcopenic based on the EWSGOP1 criteria (60% vs 16% P = 0.005). Using EWGSOP2 criteria, 50% in the hip fracture group compared to 5% in the healthy controls were sarcopenic (P = 0.002) (Table 2).

3.2. Group wise comparison (hip fracture to healthy controls): biochemistry and hormonal profile

There was no difference in corrected calcium, parathyroid hormone, and vitamin D levels between groups. Albumin levels (30.25 ± 4.71 vs 40 ± 2.58 g/L, P < 0.001), free T3 (3.61 ± 0.73 vs 4.93 ± 0.74 pmol/L, P < 0.001), and free testosterone levels by mass spectrometry were significantly lower in the hip fracture group (5.00 ± 2.07 vs 8.85 ± 3.28 nmol/L, P-value = 0.001).

Women with hip fractures had comparatively lower serum IGF-1 (11.08 ± 4.11 vs 18.75 ± 5.48 nmol/L, P-value < 0.001), Growth Hormone (1.40 ± 1.75 vs 3.00 ± 3.31 ug/L, P = 0.065) and IGF Binding Protein 3 levels (IGFBP-3) (73.57 ± 28.08 vs 121.37 ± 28.60 nmol/L, P-value < 0.001) (Table 3a).

Elevated fasting glucose levels (6.04 ± 1.12 vs 5.43 ± 0.37 mmol/L, P = 0.035) and impaired beta cell function by homeostasis model assessment beta (HOMA Beta) (76.12 ± 21.72 vs 95.12 ± 29.77, P = 0.038) were detected in the hip fracture group (Table 3b). Three

Table 1
Demographics of participants.

Variable	Hip fracture (n = 20)	Healthy controls (n = 19)	P-value
Age, yr	80.70 ± 11.30	74.79 ± 8.43	0.073
Weight, kg	67.79 ± 13.32	67.57 ± 14.47	0.962
Body mass index, kg/m^2	26.27 ± 5.36	26.20 ± 4.56	0.968
Number of medication use	6 ± 4	3 ± 2	0.001
Clinical frailty scale score [a]	3 ± 2	2 ± 1	<0.001

Values are presented as mean ± standard deviation.
[a] Clinical frailty scale score: 1-Very fit, 2-Well, 3-Managing well, 4-Vulnerable, 5-Mildly frail, 6-Moderately frail, 7-Severely frail, 8-Very Severely frail, 9-Terminally ill.

Table 2
Skeletal muscle mass (muscle cross sectional area on CT and bioelectrical impedance analysis) and function (grip strength), and sarcopenia prevalence (by EWSGOP criteria).

Variable	Hip fracture (n = 20)	Healthy controls (n = 19)	P-value
Muscle cross sectional area: non operated leg or average reading of both legs combined, cm^2	82.82 ± 16.61	100.01 ± 19.09	0.005
Janssen skeletal muscle mass, kg	16.60 ± 3.49	17.70 ± 3.74	0.349
Dry lean mass by BIA, kg	5.78 ± 3.60	6.87 ± 2.52	0.279
Lean mass by DXA, kg	n = 12 36.85 ± 4.75	n = 19 38.18 ± 6.52	0.521
Grip strength, kg	14.01 ± 6.83	22.93 ± 5.94	<0.001
Sarcopenia by EWGSOP 1 criteria†	12 (60)	3 (16)	0.005
Sarcopenia by EWGSOP 2 criteria*	10 (50)	1([5)	0.002

Values are presented as mean ± standard deviation or number (%).
BIA, bioelectrical impedance analysis; DXA, dual energy X-ray absorptiometry; EWGSOP, European Working Group on Sarcopenia in Older People.
† Sarcopenia defined by EWGSOP 1 criteria: low skeletal muscle mass by BIA (skeletal muscle mass index < 6.75 kg/m^2) combined with low grip strength (<20 kg).
* Sarcopenia defined by EWGSOP 2 criteria: low skeletal muscle mass by BIA (skeletal muscle mass index < 6.75 kg/m^2) combined with low grip strength (<16 kg).

Table 3a
Comparison of biomarkers (biochemistry and hormonal levels) between groups.

Variable	Hip fracture (n = 20)	Healthy controls (n = 19)	P-value
Corrected calcium, mmol/L	2.43 ± 1.45	2.40 ± 0.63	0.388
Albumin, g/L	30.25 ± 4.71	40.00 ± 2.58	<0.001
Parathyroid Hormone, pmol/L	6.38 ± 3.69	6.56 ± 2.56	0.858
Vitamin D, nmol/L	58.70 ± 33.56	67 ± 23.10	0.373
Thyroid stimulating hormone, mU/L	2.35 ± 2.27	2.45 ± 2.08	0.890
Free T4, pmoL/L	18.13 ± 3.66	16.62 ± 2.78	0.157
Free T3, pmol/L	3.61 ± 0.73	4.93 ± 0.74	<0.001
Free testosterone, nmol/L	5.00 ± 2.07	8.85 ± 3.28	0.001
IGF-1, nmol/L	11.08 ± 4.11	18.75 ± 5.48	<0.001
Growth Hormone, ug/L	1.40 ± 1.75	3.00 ± 3.31	0.065
IGFBP-3, nmol/L	73.57 ± 28.08	121.37 ± 28.60	<0.001
IGF-1/IGFBP-3 ratio	0.17 ± 0.11	0.16 ± 0.03	0.657

Values are presented as mean ± standard deviation.
IGF-1, insulin-like growth factor-1; IGFBP-3, insulin-like growth factor binding protein-3.

women in this group were on therapy for diabetes; 2 on insulin and the other on a combination of metformin and a dipeptidyl peptidase 4 inhibitor. After exclusion individuals who were on insulin secretagogue and insulin treatment from the analysis, the reduced beta cell function observed in the hip fracture group was still maintained.

3.3. Regression analysis on the whole study population: hormonal variables to muscle mass and strength

Univariate regression analysis on the whole study population was performed to assess the relationship between variables which were significantly different between groups to skeletal muscle mass (muscle cross sectional area on CT) and muscle function (grip strength). To correct for the effect of age, regression analysis was also performed adjusting for age as a confounder. After correction for age, serum IGF-1, IGFBP-3 levels and HOMA beta were associated with skeletal muscle area on CT and grip strength (Table 4).

3.4. Comparison sarcopenic to non-sarcopenic women: biochemistry and hormonal profile

The whole population was divided into the sarcopenic and non-sarcopenic group by EWGSOP2 criteria for comparison of hormonal profile between sarcopenic to non-sarcopenic women. Albumin and serum IGFBP-3 levels were significantly lowered in the sarcopenic group ($P < 0.05$). There was a trend towards lower free testosterone levels, serum IGF-1 levels, and HOMA beta in the sarcopenic group. However, the difference between groups in these variables did not reach statistical significance ($P > 0.05$) (Table 5a). There was no significant difference detected in glucose or insulin levels when groups were divided by sarcopenia criteria by EWGSOP2 (Table 5b).

3.5. Binary logistic regression: hormonal variables to sarcopenia status

Binary logistic regression was performed on the whole population assessing the relationship between hormonal variables to sarcopenia status adjusted for age by EWGSOP1 criteria. For completion, analysis was also performed using the EWGSOP2 criteria. Results are reported as the odds of developing sarcopenia for every unit reduction in the independent variable.

When the population was assessed using the EWGSOP 1 criteria, insulin was the only variable which was significantly associated with sarcopenia status. For every 1mu/L decrease in insulin levels, the odds of developing sarcopenia increased by a factor of 1.40 (OR 1.40, P-value = 0.045) (Table 6). When the population was assessed using the EWGSOP2 criteria, age was a stronger predictor of sarcopenia.

4. Discussion

This study was performed on older women with hip fractures and were compared to healthy women from the community. Consistent with previous studies, there was a significant proportion of sarcopenic women in the hip fracture group. This was further supported by the presence of low muscle cross sectional area on mid-thigh CT and reduced grip strength. This confirms that women with hip fractures are a good model for sarcopenia in older women.

Low albumin levels, a marker of nutritional state were observed in the hip fracture group. These findings were consistent with other studies [35], and suggests impaired nutrition may be a risk factor for hip fracture. Alternatively, loss of albumin from inflammation and leakage into the extravascular space can occur in critically ill patients [36], and could be another reason for the low albumin levels seen in the hip fracture group.

Insulin-like growth factor-1 (IGF-1) positively affects skeletal muscle growth through its interaction and activation of the

Table 3b
Comparison of fasting glucose, insulin and beta cell function between groups.

Variable	Hip fracture (n = 17) †	Healthy controls (n = 19)	P-value
Fasting glucose, mmol/L	6.04 ± 1.14	5.43 ± 0.37	0.035
Fasting insulin, mU/L	9.58 ± 5.19	10.37 ± 5.86	0.663
HOMA Beta	76.12 ± 21.72	95.12 ± 29.77	0.038

Values are presented as mean ± standard deviation.
HOMA, Homeostasis Model Assessment.
† Individual of insulin secretagogue and insulin excluded from analysis.

Table 4
Univariate linear regression analysis with muscle area on CT and grip strength as dependent variables. β Analysis adjusted for age as a confounder.

Outcome variable	Independent variable	Unadjusted model		Adjusted model corrected for age β	
		Adjusted r^2	P-value (95% confidence interval)	Adjusted r^2	P-value (95% confidence interval)
Muscle area on CT, cm^2	Serum albumin	0.11	0.022 (0.17, 2.14)	0.11	0.059 (−0.03, 2.06)
	Serum IGF-1	0.17	0.007 (0.40, 2.33)	0.16	0.017 (0.24, 2.26)
	IGFBP-3	0.22	0.003 (0.09, 0.43)	0.21	0.013 (0.05, 0.43)
	HOMA Beta	0.31	<0.001 (0.21, 0.61)	0.34	<0.001 (0.21, 0.61)
	Free testosterone	0.19	0.006 (0.86,4.81)	0.19	0.018 (0.46, 4.58)
Grip Strength, kg	Serum albumin	0.34	<0.001 (0.41, 1.08)	0.61	0.001 (0.23, 0.77)
	Serum IGF-1	0.18	0.004 (0.20, 0.95)	0.55	0.012 (0.09, 0.66)
	IGFBP-3	0.28	0.001 (0.05, 0.19)	0.60	0.026 (0.01, 0.12)
	HOMA Beta	0.08	0.051 (0.00, 0.18)	0.56	0.008 (0.03, 0.15)
	Free testosterone	0.15	0.016 (0.21, 1.89)	0.58	0.078 (−0.06, 1.17)

CT, computed tomography; IGF-1, insulin-like growth factor-1; IGFBP-3, insulin-like growth factor binding protein-3; HOMA, Homeostasis Model Assessment.

Table 5a
Comparison of biomarkers (biochemistry and hormonal levels) between sarcopenic and non-sarcopenic groups.

Variable	Sarcopenic (n = 11)	Non Sarcopenic (n = 28)	P-value
Corrected calcium, mmol/L	2.43 ± 0.16	2.41 ± 0.09	0.666
Albumin, g/L	30.73 ± 6.17	36.68 ± 5.47	0.005
Parathyroid Hormone, pmol/L	7.44 ± 3.52	6.09 ± 2.97	0.234
Vitamin D, nmol/L	58.46 ± 38.00	64.43 ± 25.07	0.638
Thyroid stimulating hormone, mU/L	3.33 ± 2.68	2.03 ± 1.84	0.093
Free T4, pmol/L	18.74 ± 3.67	16.87 ± 3.05	0.113
Free T3, pmol/L	3.66 ± 0.86	4.49 ± 0.95	0.017
Free testosterone, nmol/L	5.60 ± 1.70	7.08 ± 3.67	0.234
IGF-1, nmol/L	12.09 ± 5.14	15.89 ± 6.26	0.082
Growth Hormone, ug/L	1.32 ± 1.58	2.52 ± 3.00	0.220
Insulin like Growth Factor Binding Protein 3, nmol/L	69.94 ± 28.27	104.24 ± 35.57	0.011
IGF-1/IGFBP3 ratio	0.17 ± 0.05	0.17 ± 0.10	0.879

Values are presented as mean ± standard deviation.
IGF-1, insulin-like growth factor-1; IGFBP-3, insulin-like growth factor binding protein-3.

Table 5b
Comparison of fasting glucose, insulin and beta cell function between sarcopenic and non-sarcopenic groups.

Variable	Sarcopenic (n = 11)	Non Sarcopenic † (n = 25)	P-value
Fasting glucose, mmol/L	5.87 ± 0.68	5.68 ± 0.97	0.549
Fasting insulin, mU/L	8.64 ± 3.44	10.52 ± 6.09	0.343
HOMA Beta	75.06 ± 22.93	91.02 ± 28.53	0.112

Values are presented as mean ± standard deviation.
† Individual on insulin secretagogue and insulin excluded from analysis.

Table 6
Binary logistic regression analysis with sarcopenia status by EWGSOP1 criteria, adjusted for age.

Outcome variable	Independent variable adjusted for age	OR	P-value (95% confidence interval)
Sarcopenia Status (EWGSOP1)	Serum albumin	1.16	0.061 (0.99, 1.34)
	Serum IGF-1	1.05	0.549 (0.90, 1.22)
	IGFBP-3	1.02	0.186 (0.99,1.06)
	HOMA Beta	1.01	0.377 (0.98, 1.05)
	Free testosterone	1.31	0.216 (0.86, 2.01)
	Serum insulin levels	1.40	0.045 (1.01, 1.93)

EWGSOP, European Working Group on Sarcopenia in Older People.
Sarcopenia defined by EWGSOP 1 criteria: low skeletal muscle mass by BIA (skeletal muscle mass index < 6.75 kg/m^2) combined with low grip strength (<20 kg).
IGF-1, insulin-like growth factor-1; IGFBP-3, insulin-like growth factor binding protein-3; HOMA, Homeostasis Model Assessment.

mammalian target of rapamycin (mTOR) pathway. Unbound IGF-1 has a short half-life, and thus the majority of IGF-1 in the circulation is bound to IGF binding proteins [37]. IGF binding protein 3 (IGFBP-3), being the most abundant in the circulation, was measured in our study to provide further information. In our cohort, IGF-1 and IGFBP-3 were both positively related to skeletal muscle mass and function, suggesting a stimulatory effect on muscle growth. These findings combined with lowered IGF-1 and IGFBP-3 levels observed in the our hip fracture group were similar to findings from previous studies in hip fracture [38] and stroke patients [39]. Additionally, animal models have observed the protective effect of IGF-1 and IGFBP-3 on the muscle [40], whilst cell culture models suggests IGFBP-3 upregulates the mTOR pathway [41]. The positive relationship observed between IGF-1, IGFBP-3 to skeletal muscle mass and function may suggest a protective effect of raised IGF-1 and IGFBP-3 against sarcopenia, although this requires further study.

Free testosterone levels by mass spectrometry were positively associated with skeletal muscle mass and strength and were observed to be lowest in our hip fracture cohort. Using DXA and bioelectrical impedance analysis, the positive relationship between elevated free testosterone levels and lean body mass have been reported before [17,42]. Our analysis strengthens this finding further by showing the positive association between free testosterone levels to skeletal muscle mass on CT, a gold standard imaging tool for sarcopenia.

There was a positive relationship between free testosterone to grip strength. However, this relationship became non-significant after adjustment for age, suggesting that there were other factors contributing to the loss of muscle function with age. In this setting, free testosterone levels was a better indicator of low skeletal muscle mass but not reduced function.

Another finding in our study is that of lowered beta cell function by the homeostasis model, an indicator of intrinsic insulin production in the hip fracture group. Insulin is secreted by the pancreatic beta cells and works by enhancing glucose uptake into the target cells through its effect on the glucose transporter. Insulin appears to have several roles; glucose homeostasis and protein synthesis, and likely has an indirect effect on the bone. In muscle, in the presence of amino acids, insulin prevents protein breakdown and increases protein synthesis through activation of the mTOR 1 signalling [43–45]. In our study HOMA beta remained significantly associated with skeletal muscle area and function independent of age. This finding combined with the positive association between insulin to sarcopenia status further supports the role of insulin on the skeletal muscle.

When a comparison of hormonal levels was performed with the population divided into sarcopenic and non-sarcopenic individuals by EWGSOP2 criteria, IGFBP-3 and albumin levels were significantly lower in sarcopenic women. While low levels of albumin are

indicative and consistent with sarcopenia, low IGFBP-3 detected in sarcopenic women supports a likely link with the skeletal muscle as highlighted earlier in the discussion.

Analysis by binary logistic regression assessing the relationship between hormonal variables to sarcopenia status was performed using EWGSOP 1 and 2 criteria. When adjusted for age using EWGSOP1 criteria, insulin was associated with increased odds of sarcopenia. However, this relationship became non-significant when analysis was performed using EWGSOP2 criteria indicating age as a stronger predictor of sarcopenia status by EWGSOP2 criteria.

Another alternative explanation is that the EWGSOP2 criteria may underestimate sarcopenia in our population. Amendments to grip strength criteria in EWGSOP2 was made based on a study [46] which was performed in populations in the United Kingdom. To our knowledge at present there is no normative data for grip strength for the Australian population. Villani [47] recently compared both EWGSOP criteria in an Australian population. They report a significant discordance between both definitions of sarcopenia. Given this finding further studies are needed to further evaluate and compare both EWGSOP 1 and 2 criteria in the Australian population.

To summarise our study findings, women with hip fractures have a high prevalence of sarcopenia. To our knowledge, impaired beta cell function and low free testosterone levels have not been reported in the hip fracture cohort and serves as new knowledge. Whilst low IGF-1 and IGFBP-3 levels been reported in hip fracture patients before, the mechanisms underlying these findings requires further research.

Our study had several limitations. We were unable to age match our study participants, hence limiting direct comparison between groups. Despite this, our observations can still shed light on mechanisms of skeletal muscle loss with age. Blood samples for patients with hip fracture patients were collected between day 1–5 post-operatively, potentially confounding our results with the effects of acute surgical stress. We did not have follow up blood samples for hormonal analysis which would allow further comparison of hormone levels in the non-stressed state. Our lack of DXA data limits our ability to use this as a measure of sarcopenia in our cohort and therefore further analysis. It is possible that those who were able to return for their DXA scans post-operatively were less frail and less sarcopenic. This thus explains the lack of difference seen in skeletal muscle mass between groups by DXA data. Although dry lean mass measurement by bioelectrical electrical impedance analysis were available for all participants, we were unable to show a statistically significant difference between groups. This likely relates to our small sample size further limiting the generalisability of our study findings.

Further work would involve exploring the relationship between the IGF-1 pathway, insulin homeostasis and its effect on skeletal muscle regulation in these women.

5. Conclusions

Women with hip fractures have a high prevalence of sarcopenia. Low serum IGF-1, IGFBP-3, free testosterone and impaired beta cell function were detected in these women and may serve as potential biomarkers for sarcopenia. This is an area for future research.

Conflicts of interest

This study was funded by Monash University Victoria Australia and the Elaine and Frank Derwent Memorial Research Grant Eastern Health Foundation, Victoria Australia reference EHFRG2017_002. No other potential conflict of interest relevant to this article was reported.

CRediT author statement

Ming Li Yee: Formal analysis, Resources, Writing - Original Draft, Writing - Review & Editing. **Raphael Hau**: Resources. **Alison Taylor**: Resources. **Mark Guerra**: Resources. **Peter Guerra**: Resources. **Peteris Darzins**: Resources. **Christopher Gilfillan**: Conceptualization, Methodology, Formal analysis, Writing - Original Draft, Writing - Review & Editing, Supervision.

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Chapter 4: Sex steroid profile and histology of muscle biopsies for fibre type distribution and centralised muscle nuclei in women with hip fracture compared to controls.

4.1 Abstract

In chapter 3, free testosterone levels by liquid chromatography mass spectrometry (LCMS) were lowest in women with hip fracture compared to women in the other groups. As these women were confirmed to have low skeletal muscle mass and grip strength, the role of androgens in skeletal muscle was further examined.

This chapter expands on chapter 3 by exploring the full sex steroid profile particularly the 11-oxygenated (11-OXO) androgens produced by the adrenal gland and their relationship to skeletal muscle mass and strength. As androgens increase satellite cell numbers in older men and therefore promote muscle regeneration [152] the relationship between androgen to the presence of centralised muscle nuclei as a surrogate for muscle fibre regeneration was explored. Evidence in the literature also suggest oestrogen may have a positive effect on the skeletal muscle [385, 386]. Therefore as a secondary analysis, the relationship between oestradiol and oestrone levels to muscle mass was examined.

Key findings from this study are low levels of 11-OXO androgens detected in the hip fracture group. In contrast to findings in men, our study demonstrate the presence of increased skeletal muscle central nuclei in women with low androgen levels providing evidence that skeletal muscle regeneration is a response to low androgen levels in these women. An unexpected finding were high serum oestradiol levels in the hip fracture group. The significance of this remains uncertain but may indicate increased aromatisation in acute illness.

4.2 Introduction

Background

Steroids are divided into different groups dependent on the number of carbons present [387]. Androgens are a group of C19 steroids containing 19 carbons and are the most abundant sex steroids in men and women. Androgen production in the adrenal gland is driven by adrenocorticotrophic hormone (ACTH). In contrast, in the reproductive system, androgen synthesis occurs in ovaries in women and testes in men [388] under the influence of luteinizing hormone (LH). A key role of androgens in men is to induce secondary sexual characteristics, while in women androgens primarily act as precursors to oestrogen synthesis.

Androgen production

Steroid production [387] begins with the transfer of cholesterol from the outer to inner mitochondrial membrane by the action of steroidogenic acute regulatory protein [389]. Cleavage of its side chain leads to formation of pregnenolone, further metabolized to form 17 α-hydroxy pregnenolone (17OH-PREG). 17OH-PREG can act as a precursor to form dehydroepiandrosterone (DHEA) and further converted to androstenedione (A4) and subsequently testosterone [389]. In the presence of aromatase, androstenedione (A4) is converted to oestrone, and testosterone into oestradiol (Figure 4.2a).

In the adrenal gland, A4 metabolism leads to production of the 11-oxygenated steroids forming either 11-βhydroxy androstenedione (11OH4), 11-ketoandrostenedione (11K4) and 11-ketotestosterone (11KT) [389]. 11OH4 is also a precursor to 11-keto dihydrotestosterone [390]. These groups of 11-oxygenated steroids will be the focus of this study and are highlighted in grey in Figure 4.2b.

Figure 4.2a Formation of oestrogens from C19 precursor steroids in post-menopausal women. Adapted from Wang [391].

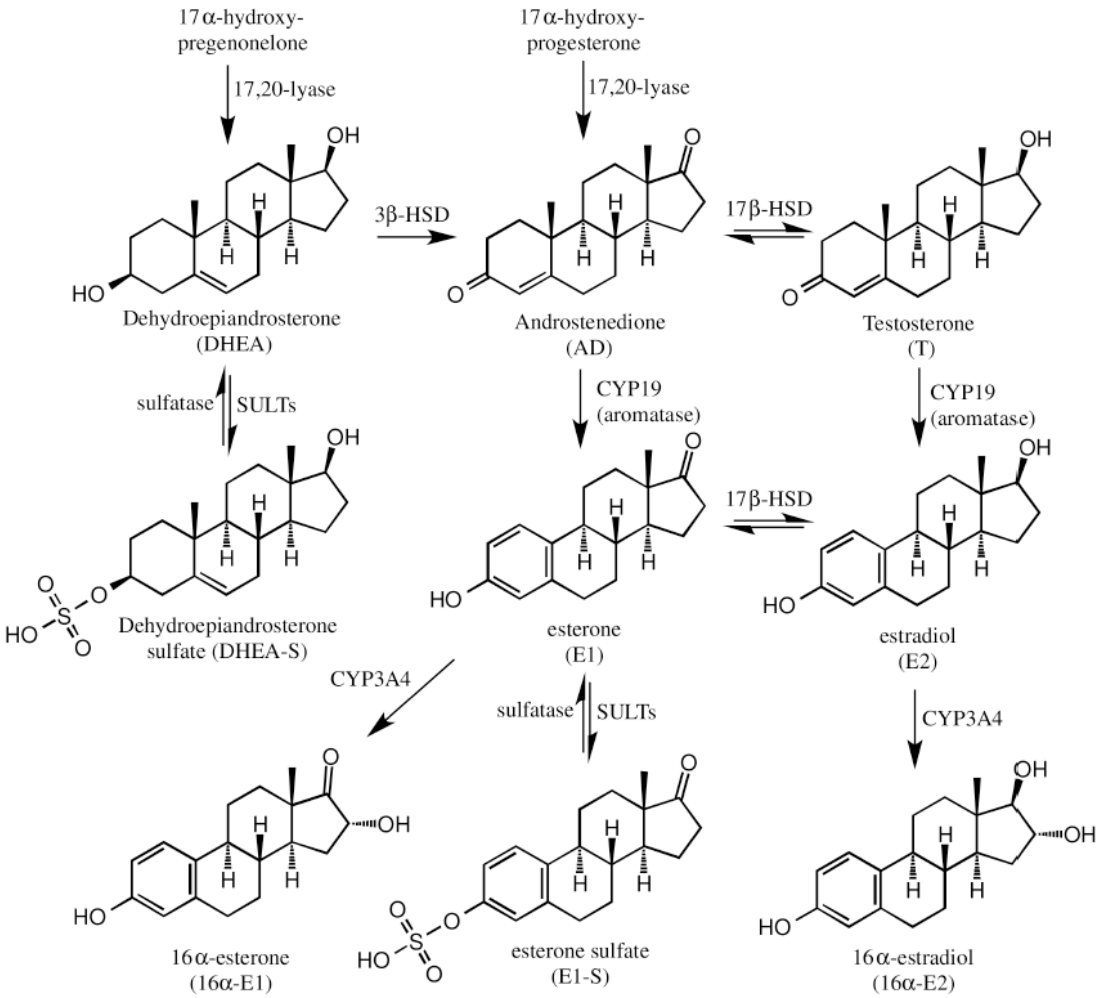
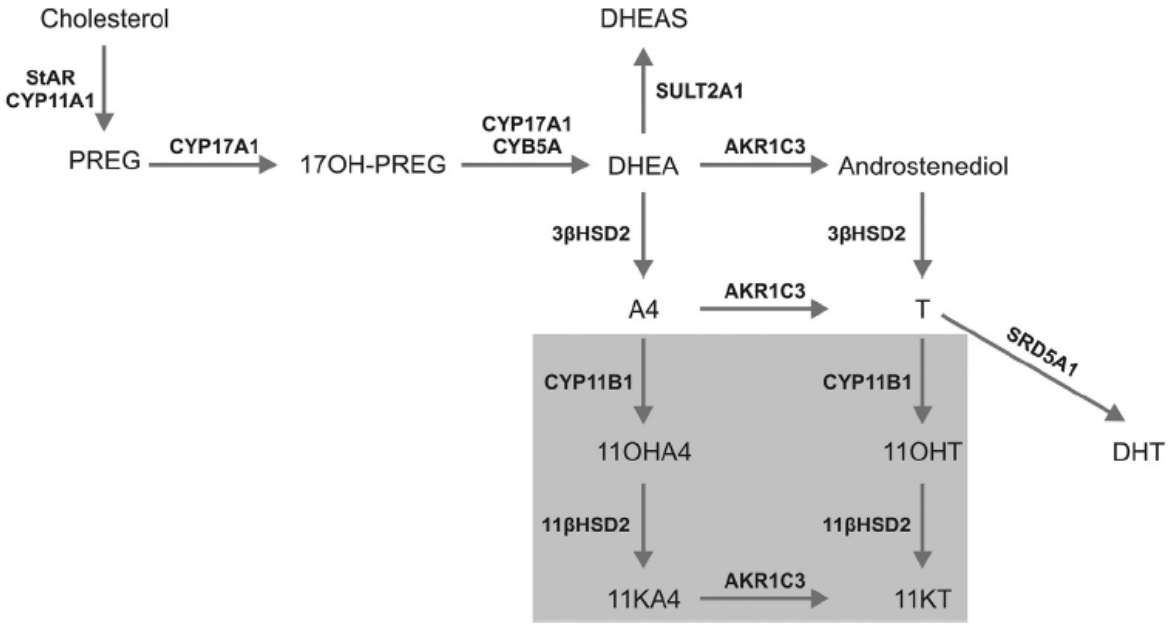


Figure 4.2b Diagram showing steroid production in the adrenal gland. Highlighted in grey are the 11 oxygenated steroids. Adapted from Pretorius [389].



Three β HSD2 (3β-hydroxysteroid dehydrogenase type 2), 11KA4 (11-ketoandrostenedione), 11-KT (11-Ketotestosterone), 110H4 (11-β Hyroxyandrostenedione), 11 OHT (11-β-hydroxy testosterone), 17-OH PREG (17 α-Hydroxypregnenolone), A4 (Androstenedione), AKR1C3 (Aldo-keto reductase 1C3), CYPB5A (Cytochrome b5), CYP11A1 (Cytochrome P450 cholesterol side chain cleavage), CYP11B1 (Cytochrome P450 11 β Hydroxylase), CYP17A1 (Cytochrome P450 17 α-hydroxylase/17-20 lyase), DHEA (Dehydroepiandrosterone), DHT (Dihydrotestosterone), PREG (Pregnenolone), SRD5A1 (Steroid 5 α reductase Type 1), StAR (Steroidogenic acute regulatory protein).

11-oxgenated steroids and its clinical relevance

The 11-oxygenated steroids consisting of 11OH4, 11KA4, 11OHT, 11KT and 11 KDHT are a group of steroids specifically produced by the adrenal glands [392]. Conventionally these group of steroids were thought to have negligible androgenic activity. In recent years, evidence suggest the 11-oxygenated steroids may be as potent as testosterone [185] and are now considered a group of major bioactive androgens in women. It is clinically relevant in several conditions with androgen excess: congenital adrenal hyperplasia and polycystic

ovarian syndrome (PCOS) [389]. The 11-oxygenated steroids have previously been reported as the predominant circulating androgens in women with PCOS [393]. Increased lean body mass [189] and muscle strength [190, 394] in these women points towards a possible link between the 11-oxygenated steroids to skeletal muscle. So far, this has not yet been studied.

Increasing age in men and women are associated with a decline in the C19 steroids [392] and can confound study findings in the older population. Conversely the 11-oxygenated androgens do not decline with age [186] and therefore serves as an attractive alternative for studies in the older population.

Androgen and muscle

The role of androgen and its anabolic effect on the muscle particularly in men is well established. Testosterone supplementation in hypogonadal men results in skeletal muscle hypertrophy and improvements in muscle strength [172, 173]. Similar dose dependent effects of testosterone were also observed in eugonadal men [174, 175] although gains in older individuals were either modest or non-sustained [176, 177].

In post-menopausal women, free testosterone and dehydroepiandrosterone (DHEA) levels were positively associated with lean body mass [187, 381] and muscle strength [188]. Testosterone administration in women were shown to increase lean body mass, muscle strength and stair climb [395], however administration of DHEA in a separate study did not result in changes in muscle strength in older women [352].

Oestrogen and muscle

The expression of oestrogen receptors (α and β) have been reported before in human skeletal muscle [386, 396]. In animal models and cell culture studies, oestrogen were

demonstrated to have an anti-apoptotic effect on the muscle through the AKT pathway [397], maintain satellite cell function, muscle regeneration [398, 399], induce myoblast growth [400], and regulate myofibre growth and regeneration [401].

In ovariectomized rats, muscle fibre cross sectional area was smaller [401] and muscle strength was comparatively weaker compared to those who were not ovariectomized or had their oestradiol levels replaced [402]. Trials in post-menopausal women evaluating the use of hormonal replacement therapy (HRT) report improved muscle strength [403] and muscle contractility [404] in HRT users. Conversely, there are also negative reports whereby HRT use have no effect on the muscle [405-407]. Therefore it still remains uncertain if oestrogen has a positive effect on the muscle particularly in older women.

Skeletal muscle nuclei

Skeletal muscles are identified by striated multinucleated myofibres and the hallmark presence of peripherally located nuclei [149]. In contrast regenerating myofibres are recognized by the presence of central nuclei [149]. Skeletal muscle myofibres develops from the activation of quiescent muscle satellite cells which lies dormant between the sarcolemma and basal lamina [147]. Activated satellite cells differentiate to form mononucleated myoblasts. Fusion of mononucleated myoblasts results in formation of multinucleated myofibres with central nuclei. As mature myofibres develops, nuclei which were initially concentrated in the central area of the myotube spread along the long axis and disperse peripherally [149]. The presence of central nuclei have been used as a marker of muscle regeneration [150]. In men, androgen increases muscle satellite cells, myonuclear number and muscle fibre size in Type I and Type II muscle fibres [152, 201-203]. Whether or not this observation holds true for women remains uncertain.

Hence for this study we aim to characterise the sex steroid profile and assess the relationship between these sex steroids to measures of skeletal muscle mass and strength. The presence of centralised skeletal muscle nuclei, muscle fibre type distribution and muscle fibre size and the relationship to sex steroid profile was also examined. We hypothesized that low levels of sex steroids would be observed in the hip fracture group, and high sex steroids levels would be associated with higher muscle mass and function as well as high muscle centralised nuclei counts and larger fibre diameters.

4.3 Aims

- To characterise the sex steroid profile in women with hip fracture compared to women awaiting hip replacement and healthy controls

- To assess the relationship between androgens to measures of skeletal muscle mass and strength in this group of women

- To assess the relationship between androgens to the presence of centralised skeletal muscle nuclei as a measure of skeletal muscle regeneration, muscle fibre type distribution and muscle fibre size

- To compare oestradiol and oestrone levels, and its relationship to centralised muscle nuclei in this group of women as a secondary analysis

4.4 Methods

Participant recruitment and selection:

The same group of women who were enrolled in Chapter 3 (Cohort 2) were consented for this study. Details regarding recruitment are reported in Chapter 3.

Peripheral blood sampling:

Peripheral blood sample was collected after an 8 hour overnight fast. In women from the hip fracture group who were enrolled following presentation to hospital, collection of peripheral blood sample was performed opportunistically together with their post-operative blood sample collection, between Day 1 to Day 5 following hip surgery. Women in the hip replacement and healthy control group had blood samples obtained prior to their surgery or muscle biopsy. Blood samples were processed and stored in -80 ° C freezer.

Androgen profile analysis by liquid chromatography mass spectrometry (LCMS):

500 µl (micro litre) of serum samples were sent for processing at the ANZAC research institute Sydney, Australia by Professor David Handelsman at his laboratory. Samples were analysed for an expanded androgen profile including the group of 11-oxygenated steroids produced by the adrenal glands.

Briefly the process of LCMS consists of high-performance liquid chromatography (HPLC) followed by mass spectrometry [408]. In HPLC the solvent (sample) is injected by a pump into a column (mobile phase) made of porous material. This is followed by the stationary phase where separation of components in the solvent occurs by adsorption and is dependent on the affinity between the different components (in the solvent) and the column.

The eluent obtained from this process undergoes ionization in the mass spectrometer and analytes are separated further by their mass/ionic charge and recorded by the detector. Identification of the different compounds of interest are determined through a chromatogram mapped using information from the retention time in the column [408], and the calculated mass to charge ratio obtained from the mass spectrometer [408].

Methods for the LCMS procedure undertaken at ANZAC research institute for the analysis of androgens are reported in reference to the methods by Skiba [409]. Processing methods by LCMS were obtained from Reena Desai, senior scientist at ANZAC research institute:

1) Aliquots of 200 µL of serum, standards and quality control samples in 5 mL glass tubes were prepared. This was fortified with 50 µl of deuterated steroid internal standards and left at 4° C (Celsius) for 15 minutes.
2) 1ml of methyl tert-butyl ether was added to the samples and mixed for 1 minute to extract the steroids. The tubes were then covered and stored at 4° C for one hour. Following phase separation, the lower aqueous layer is frozen at -80° C for 30 minutes. The upper organic layer was transferred into a clean glass tube and left overnight in a fume hood at 37 °C to allow evaporation of solvent.
3) Dried extracts were re suspended in 75 µL of 20% methanol: water and then mixed for 1 minute with 50 µL of extract transferred into a 96 well µtitre (micro titre) plate for injection into the LCMS machine.
4) The ultra-pressure liquid chromatography (LC) conditions comprised elution of steroids using a methanol/water gradient from a Kinetex Phenyl Hexyl column (100 mm X 2.1 mm X1.7 µm, part #00D-4500-AN) with a Phenomenex guard cartridge (Security Guard ULTRA Cartridges, UHPLC Phenyl for 2.1mm ID Columns, part # AJ0-8788) at a column temperature of 45 °C and flow rate of 0.35 mL/min. The eluant was introduced into the mass spectrometer without splitting and a total run time of 13 min (minutes).
5) The LC running conditions were split into three periods to match the ionization requirements of the steroids. The run times in the first period for the keto-androgens (in positive ionization mode) were 11KT (4.49 min), 11KA (4.68 min) and 11KDHT (5.14 min). Those in the second period for estrogens (in negative ionization mode) included oestradiol (5.74 min) and oestrone (6.56 min). The remainder of steroids were run in the third period (positive ionization mode) comprising 3β-diol (7.03 min), Testosterone (7.07 min), Androstenedione (7.25 min), Dehydroepiandrosterone (7.39 min)3α diol (8.64 min), Dihydrotestosterone (8.73 min) and Progesterone (10.88 min).

6) The mass spectrometer machine used was API-5000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) equipped with an atmospheric pressure photoionization source which operated in both negative and positive ion modes (probe settings X=5, Y=10, Z=7). The limits of detection, limits of quantification between run (15 replicates) and within run (10 replicates) and reproducibility are reported in Table 4.4a.
7) The limits of detection is defined as the lowest amount of analyte which can be reliably distinguished from 'analytical noise' [410]. The limits of quantification is the lowest concentration at which the analyte can be reliably detected [410]. Levels between the limits of detection and limits of quantification have a coefficient variation of no more than 20%.

Table 4.4a: Reports of limits of detection, limits of quantification and reproducibility between and within runs for the different hormones assessed by liquid chromatography mass spectrometry.

Analyte	Limits of detection (LOD)	Limits of quantification (LOQ)	Reproducibility (Between runs)	Reproducibility (Within runs)
11-Ketotestosterone (ng/ml)	0.02	0.05	3-9%	4-8%
11-Ketodihydrotestoserone (ng/ml)	0.05	0.10	5-7%	5-7%
11-Ketoandrostenedione (ng/ml)	0.02	0.05	3-8%	5-7%
Testosterone (ng/ml)	0.010	0.025	2-9%	4-8%
Dihydrotestosterone (ng/ml)	0.05	0.10	4-12%	4-9%
Dehydroepiandrosterone (ng/ml)	0.02	0.05	3-6%	8-12%
Androstenedione (ng/ml)	0.03	0.05	4-10%	6-9%
Progesterone (ng/ml)	0.02	0.05	4-7%	4-9%
Oestradiol (pg/ml)	3	5	4-8%	5-8%
Oestrone (pg/ml)	3	5	3-6%	5-9%

In the 49 women, several had undetectable levels for the analytes of interest (Table 4.4b). For cases where levels were not detectable an arbitrary value was allocated based on the

following: if the analyte was detectable in more than 80% of samples, the analysis was performed assuming the undetectable levels were half-way between zero and the lower limit of detection. If the analyte was detectable in less than 80% of samples, analysis was performed as a dichotomized variable defined as either the presence or absence of the analyte of interest (detectable/undetectable).

As only 35% of oestradiol levels by LCMS were detectable, serum oestradiol levels by immunoassay analysis were also provided. To minimize confounders, two women in the study with a history of breast cancer who were on an aromatase inhibitor or selective estrogen receptor modulator were excluded from the analysis. Final analysis was performed on serum samples from 47 women.

Table 4.4b: Proportion of undetectable levels in analytes.

Analyte	Detectable levels n (%)	Undetectable levels n (%)
11-Ketotestosterone (ng/ml)	48 (98%)	1 (2%)
11-Ketodihydrotestoserone (ng/ml)	5 (10%)	44 (90%)
11-Ketoandrostenedione (ng/ml)	49 (100%)	0 (0%)
Testosterone (ng/ml)	48 (98%)	1 (2%)
Dihydrotestosterone (ng/ml)	18 (37%)	31 (63%)
Dehydroepiandrosterone (ng/ml)	48 (98%)	1 (2%)
Androstenedione (ng/ml)	48 (98%)	1 (2%)
Oestradiol (pg/ml)	17 (35%)	32 (65%)
Oestrone (pg/ml)	47 (96%)	2 (4%)

Muscle biopsy:

Muscle biopsy was obtained from either the vastus lateralis or gluteus maximus area. Women who had hip surgery (hip fracture or hip replacement group) had muscle samples obtained from either the vastus lateralis or gluteus maximus by the orthopaedic surgeon at the time of their surgery. Muscle samples were retrieved in the operating theatre and immediately processed on site by myself.

Healthy controls who were not scheduled for surgery had their muscle biopsy performed under local anaesthesia (1% lignocaine) at the Eastern Health Clinical School research department. Muscle biopsy was obtained from the lateral aspect of the mid-thigh from the vastus lateralis using a Bergstrom muscle biopsy needle. The first few initial muscle biopsies was performed by Assoc Prof Andrew Garnham, sports physician. Subsequent procedures was performed by myself and Professor Gilfillan, assisted by the endocrine nurse. Participants were contacted on the same day of the procedure and 24 hours post procedure to monitor for any post procedure complications.

Muscle biopsy processing:

Following retrieval, the muscle samples were divided into equal parts for protein, histology and Ribonucleic (RNA) analysis. As liquid nitrogen was not directly accessible at the operating theatres, a dry shipper was utilized (as an alternative to liquid nitrogen) to snap freeze muscle samples.

Samples for RNA analysis were embedded in RNA later solution (Invitrogen) and transported in a chilled ice box until storage in a fridge at 4° C for 24 hours. Following this samples were removed from the RNA later solution and stored in -80 °C freezer. Samples for protein analysis were inserted into its storage vial and snap frozen in the dry shipper before storage in -80° C freezer. Samples for histology analysis were placed into a vial (containing

isopenthane which was pre chilled in the dry shipper), snap frozen in the dry shipper before storage in -80 °C freezer. All tissues were stored at -80 degree Celsius prior to transfer on dry ice to Deakin University Burwood Victoria Australia for laboratory analysis.

Processing of muscle samples for histology, immunohistochemistry and Haematoxylin and Eosin staining was performed by Dr Paul Della Gatta and Dr Evelyn Zacharewicz at Deakin University Burwood Melbourne Australia.

Processing of muscle biopsy samples allocated for histology analysis:

Cross sections of frozen muscle sample were cut using Leica CM 1850 Cryostat (Leica Biosystems, Vic, Aus) in a -20° Celsius chamber. Muscle samples were embedded in Tissue Tek compound, cut in 8 µm thickness and mounted onto silane-coated glass slides. Hematoxylin and Eosin (H&E) staining was performed as described in the section of histological analysis in Selathurai [411]. Frozen muscle sections were thawed then air dried at room temperature for 10 minutes. The samples were fixed in 10% buffered formalin (Grale Scientific, VIC, AUS) for 2 minutes and rinsed in tap water.

Samples were incubated in Hematoxylin (Grale Scientific) for 5 minutes. This is followed by washing in acid alcohol (0.3% concentrated hydrochloric acid (HCL) in 70% ethanol) for 5 secs, Scott's tap water (0.25% sodium bicarbonate ($NaHCO_3$) and 2.5% Magnesium sulphate ($MgSO_4$) in tap water) for 5 seconds. The samples were washed in tap water in between each change of solution, and subsequently incubated for 3 minutes in 1% alcoholic tap water.

The samples were then dehydrated in three changes of 100% ethanol for 3 min each and further cleared in three changes of xylene (Sigma) for 3 min each. When dry, the samples were mounted using distyrene, plasticiser, xylene (DPX) mountant (Sigma-Aldrich) and covered with a series one glass coverslip (Trajan, VIC, AUS). Using an E200 light microscope

(Nikon, NSW, AUS) the slides were viewed, and image captured with a DS-Fi2 camera (Nikon) and a DS-L3 viewer attachment (Nikon), at 4 and 10 X magnification.

Processing of muscle biopsy samples for muscle fibre typing

Immunofluorescence analysis of myosin heavy chain (MHC) isoform expression in muscle samples was performed according to Bloemberg and Quadrilatero [412], with minor alterations [411].

Briefly, 8 mm sections of muscle were affixed to StarFrost Silane coated Slides (ProScitech, Kirwan, Queensland, Australia) and frozen at -80°C. On the day of staining, slides were thawed at room temperature for 10 min and were then blocked in 10% goat serum (Life Tech) in PBS for 1 hour at room temperature. Slides were then incubated in a cocktail of primary antibodies for 1 hour at room temperature (BA-F8 (1:20; MHCI), SC-71 (1:50; MHCIIa), 6H1 (1:20, MHCIIx), Developmental Studies Hybridoma Bank, University of Iowa, Iowa, IA, USA; laminin (1:100) (L9393), Sigma Aldrich).

After washing (3 x 5 min in PBS), sections were exposed to fluorescent conjugated secondary antibodies (Alexa Fluor goat anti mouse IgG2b 647, Alexa Fluor goat anti mouse IgG1 488, Alexa Fluor goat anti mouse IgM 555, Alexa Fluor goat anti rabbit IgG H + L 405, all 1:500 (Life Technologies) diluted in 10% goat serum in PBS for 1 hour at room temperature in the dark. Following an additional 3 washes (5 min in PBS), sections were mounted in Prolong Gold (Life Technologies, Australia) and cover-slipped. Slides were visualised using an Olympus Fluoview FV10i confocal microscope (Olympus, Australia).

All images were taken using the same laser and sensitivity settings. Images of the entire muscle were taken at 10x magnification (7-12 images total) and stitched using Image J. Resulting images were analysed using Olympus Cellsens Dimensions Imaging Software. A macro was developed to semi automate the analysis. Muscle cell measurements were

obtained by quantifying the area enclosed by laminin staining and then muscle fibre type was determined by quantifying Mean Colour Intensity value. All images were cross checked by the operator and any errors were corrected manually.

Identification of muscle central nuclei from histology slides:

Thirty-nine histology slides were available for the purpose of this analysis (17 hip fracture, 7 hip replacement, 15 healthy controls). Identification of muscle nuclei were performed using Image J software (Fiji). Muscle nuclei are identified by its dark blue appearance. To reduce bias, skeletal muscle nuclei count was performed by an assessor blinded to the participant's steroid levels, group category (hip fracture, hip replacement, healthy controls) and were cross checked.

Due to the presence of multiple nuclei per skeletal muscle fibre, nuclei positioning was categorised as central (if any central nuclei was observed), intermediate (if a central nuclei was not observed but an intermediate nuclei was observed) and peripheral (if a central and intermediate nuclei was not observed, and only peripheral nuclei were observed) for each muscle fibre. Muscle fibres without nuclei in the section were excluded. (Figure 4.4a). Mean nuclei from each category were used as threshold to dichotomize the participant into high or low nuclei count.

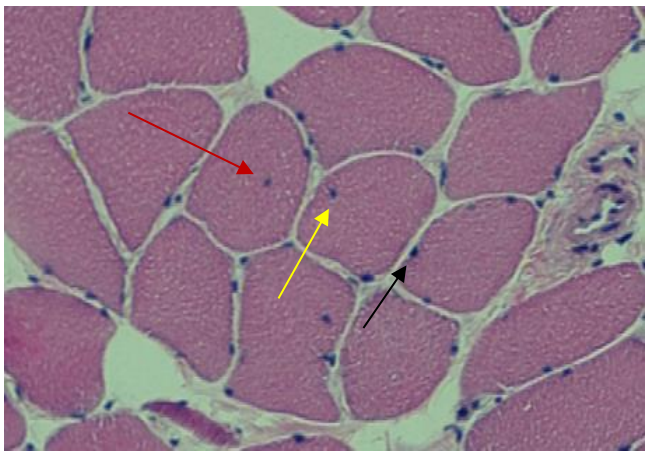


Figure 4.4a: Histology section showing nuclei location

Red arrow: Central nuclei

Yellow arrow: Intermediate nuclei

Black arrow: Peripheral nuclei

Data reporting and statistical analysis:

Parametric data are reported as means ± standard deviation, while non-parametric data are reported as median (interquartile range). A comparison of mean or median between the three groups of women were performed using ANOVA or Kruskal Wallis, with Student's T test or Mann Whitney U test to detect differences between two groups of women. In some variables without a normal distribution, a log transformation was performed to enable mean comparison by parametric tests. Chi square test was used to compare dichotomized variables between groups. In some variables where age was considered to be a confounder ANCOVA analysis was performed to adjust for age.

Student's T test were used to compare mean androgen and oestrone levels between those with low and high nuclei count. Curve fitting suggest an exponential relationship between centralised nuclei count and androgen levels. Therefore a regression analysis using an exponential function was performed.

Univariate linear regression analysis was performed assessing the relationship between the different analytes to measures of skeletal muscle mass and strength. The relationship to sarcopenia status defined by EWGSOP 1 criteria [33] was also assessed in a binary logistic regression. p value <0.05 was considered statistically significant. All analysis was performed using SPSS version 26.

4.5 Results:

4.5.1 Demographics:

Participants in this study were the same group of women in Chapter 3 (Cohort 2). Demographics of these women were previously reported. For convenience, this is repeated in Table 4.5.1a.

Table 4.5.1a Demographics of study participants.

Category	**Hip fracture (n=19)**	**Hip replacement (n=9)**	**Healthy controls (n=19)**	**p value**
Age (Years)	81.2 ± 11.0 †	73.0 ± 5.9 †	74.8 ± 8.4	0.05
Weight (kilograms)	68.7 ± 14.7	78.7 ± 13.7	67.6 ± 14.5	0.19
Body mass index (kg/m^2)	25.3 (23.7,31.0)	25.0 (26.3, 29.7)	25.6 (23.6, 29.1)	0.26
Medication amount	6 (3,10) *	7 (4,8)	2 (1,4) *	<0.01
Clinical Frailty Scale Score [a]	3 (2, 5) *	3 (2, 3)	2 (1, 2) *	<0.01

[a]Clinical frailty scale score: 1-Very fit, 2-Well, 3-Managing well, 4-Vulnerable, 5-Mildly frail, 6-Moderately frail, 7-Severely frail, 8-Very Severely frail, 9-Terminally ill.

* p<0.05 hip fracture to healthy controls

† p<0.05 hip fracture to hip replacement

4.5.2 Groupwise comparison:

4.5.2.1 Androgen comparison:

Groupwise comparison of androgens are reported in Table 4.5.2.1a. Serum testosterone, dehydroepiandrosterone (DHEA), 11-ketotestosterone (11-KT) and 11-ketoandrostenedione (11-KA) were significantly lower in the hip fracture group compared to healthy controls.

Androgens in the hip replacement group did not differ significantly from the healthy controls. DHEA and 11-KT levels in women with hip replacement were significantly higher than women in the hip fracture group (Table 4.5.2.1a)

Table 4.5.2.1a: Groupwise comparison androgen levels between groups.

Category	**Hip fracture (n=18)**	**Hip replacement (n=9)**	**Healthy controls (n=19)**	**p value**
Testosterone (T) ng/ml	0.08 (0.06,0.11) [a]	0.09 (0.09,0.17)	0.17 (0.12,0.28) [a]	<0.01
Dihydrotestosterone (DHT) ng/ml (yes/no) †	1 (5.3%)	2 (22.2%)	2 (10.5%)	0.40
Dehydroepiandrosterone (DHEA) ng/ml	0.19 (0.13,0.37) [ab]	0.38 (0.22,0.81) [b]	0.75 (0.47,1.16) [a]	<0.01
Androstenedione (A4) ng/ml	0.20 ± 0.14	0.24 ± 0.13	0.28 ± 0.11	0.12
11 Oxygenated steroids				
11-ketotestosterone (11-KT) ng/ml	0.19 (0.13,0.23) [ab]	0.37 (0.25,0.56) [b]	0.45 (0.36,0.62) [a]	<0.01
11-Ketodihydrotestosterone (ng/ml) (yes/no) †	5 (26.3%)	3 (33.3%)	9 (47.4%)	0.41
11-ketoandrostenedione (11-KA4) ng/ml	4.5 (0.81,2.00) [a]	1.54 (1.18,1.82)	2.00 (1.69,2.39) [a]	0.03

[a] p<0.001 hip fracture compared to healthy controls

[b] p<0.05 hip fracture compared to hip replacement

† Reporting of data as number of detectable levels (%)

4.5.2.2 Gonadotrophins, oestradiol and oestrone comparison:

This is summarized in Table 4.5.2.2a. Gonadotrophins in the hip fracture group were significantly lower in women with hip fracture compared to women in the other groups. Oestradiol levels by immunoassay were significantly higher in the hip fracture group compared to hip replacement and healthy controls. Dichotomized oestradiol levels by LCMS indicate oestradiol were more often detectable in women with hip fracture compared to women in the hip replacement group.

Oestrone was higher in the hip fracture group. As oestrone can be converted from Androstenedione (A4) a ratio was calculated and log transformation performed to allow

comparison by t-test. Both oestrone and log (Oestrone:A4) were significantly higher in women with hip fracture compared to women in the other groups.

In women with hip replacement, oestrone levels were lower than the hip fracture group while log Oestrone: A4 levels were lower than healthy controls.

Table 4.5.2.2a Groupwise comparison gonadotrophin, oestradiol and oestrone levels between groups

Category	**Hip fracture (n=18)**	**Hip replacement (n=9)**	**Healthy controls (n=19)**	**p value**
Luteinising Hormone (LH) IU/L	12.46 ±15.17 [a b]	30.71 ± 16.19 [b]	29.97 ± 10.85 [a]	<0.01
Follicle Stimulating Hormone (FSH) IU/L	33.90 ± 23.74 [a b]	67.73 ± 26.36 [b]	65.98 ± 18.82 [a]	<0.01
Oestradiol (E2) pg/ml by LCMS (yes/no) †	10 (55.6%) [b]	1 (11.1%) [b]	5 (26.3%)	0.04
Oestradiol (E2) IU/L by immunoassay	61.68 ± 77.76 [ab]	13.00 ± 39.00 [b]	4.05 ± 9.76 [a]	<0.01
Oestrone (E1) pg/ml	58.0 (28.0,81.0) [b]	24.0 (18.0,33.0) [bc]	39.0 (30.5,54.5) [c]	0.03
Log (Oestrone: A4) ratio	2.47 ±0.41 [ab]	2.05 ±0.20 [bc]	2.19 ±0.15 [ac]	<0.01

[a] p<0.001 hip fracture compared to healthy controls

[b] p<0.05 hip fracture compared to hip replacement

[c] p<0.05 hip replacement compared to healthy controls

† Reporting of data as number of detectable levels (%)

As the groups differed by age, ANCOVA analysis was performed adjusting for age as a confounder for the changes observed in gonadotrophins and oestradiol levels. Age adjusted estimated mean levels for each group are listed in Table 4.5.2.2b. Age adjusted mean levels suggest low gonadotrophins and high oestradiol levels in the hip fracture group compared to women in the hip replacement and healthy controls was confirmed independent of age.

Similarly there remains a significant difference between these groups of women in log (oestrone:A4) after adjusting for age.

Table 4.5.2.2b ANCOVA estimated means for gonadotrophins and oestradiol after adjusting for age

Age adjusted mean				
Mean ± standard error	**Hip fracture (n=18)**	**Hip replacement (n=9)**	**Healthy controls (n=19)**	**p value**
Luteinising Hormone (LH) IU/L	14.44±3.15	28.80±4.48	28.91±3.07	<0.01
Follicle Stimulating Hormone (FSH) IU/L	36.98±5.15	64.75±7.32	64.32±5.00	<0.01
Oestradiol (E2) IU/L by immunoassay	52.85±11.82	21.57±16.80	8.83±11.49	0.04
Log (Oestrone: A4) ratio	2.43 ± 0.07	2.09 ± 0.09	2.21 ± 0.06	0.02

4.5.3 Univariate regression analysis

4.5.3.1 Androgen, oestradiol and oestrone levels to skeletal muscle mass and grip strength

Univariate regression analysis was performed evaluating the relationship between the different androgens, oestradiol and oestrone to skeletal muscle mass measured by CT and DEXA. There were no significant relationships detected. A similar analysis was performed with grip strength as the independent variable. DHEA ($p<0.01$) and 11-ketotestosterone were positively associated with skeletal muscle strength ($p=0.015$) while serum oestrone and log oestrone:A4 levels was negatively associated with grip strength (Table 4.5.3.1a).

Table 4.5.3.1a Univariate regression analysis between androgens, oestradiol and oestrone to grip strength (kg).

Category	Beta (95% CI)	r^2	p value
Dehydroepiandrosterone (DHEA) ng/ml	7.03 (2.55,11.50)	0.182	<0.01
11-ketotestosterone (11-KT) ng/ml	12.67 (2.53, 22.81)	0.123	0.02
Oestrone (E1) pg/ml	-0.10 (-0.17, -0.03)	0.167	<0.01
Log (oestrone:androstenedione ratio)	-10.02 (-16.34, -3.70)	0.185	<0.01

4.5.3.2 Binary logistic regression with sarcopenia status

Binary logistic regression analysis was performed assessing the relationship between androgens, oestradiol and oestrone to sarcopenia status defined by the EWGSOP 1 criteria. Oestrone was the only variable significantly associated with sarcopenia status (OR 1.03, p value 0.02). For every 1 pg/mL increase in oestrone levels, the odds of being sarcopenic increases by 3% (Table 4.5.3.2a).

Table 4.5.3.2a: Binary logistic regression analysis with sarcopenia status.

Category	r^2	Odds ratio	p value (95% CI)
Oestrone (E1) pg/ml	0.15	1.03	0.02 (1.01,1.06)

4.5.4 Comparison sex steroid levels between groups of high and low skeletal muscle centralised nuclei count

For this analysis, the presence of central and intermediate nuclei was used as markers of skeletal muscle regeneration. A comparison of mean sex steroid levels was performed with the groups of women dichotomized into high and low nuclei counts. Mean nuclei counts for each category were used as threshold levels to define high or low nuclei counts. Results are presented in Table 4.5.4a for central nuclei:total muscle fibre ratio and Table 4.5.4b for combined central and intermediate nuclei:total muscle fibre ratio.

Serum testosterone and 11-Ketodihydrotestosterone (11-KDHT) were the two androgens observed to be consistently different in between groups of high and low nuclei count. The presence of high central nuclei and combined (central and intermediate) nuclei to total muscle fibre ratio, contrary to expectations, was associated with lower levels of testosterone and 11-KDHT. Oestrone levels was associated with high central nuclei counts.

Table 4.5.4a Comparison androgen, oestradiol and oestrone levels by nuclei count categorised by high/low groups (central nuclei).

Hormonal variable	**Mean difference**	**Standard error**	**p value (95% CI)**
Central nuclei to total muscle fibre ratio			
11-Ketotestosterone (11-KT) ng/ml	-0.05	0.07	0.49 (-0.19, 0.09)
11-Ketodihydrotestosterone (11-KDHT) (yes/no)	-0.14	0.07	0.04 (-0.28, -0.01)
11-Ketoandreostenedione (11KA4) ng/ml	-0.39	0.33	0.25 (-1.07, 0.29)
Testosterone ng/ml	-0.07	0.02	0.01(-0.11, -0.02)
DHEA	-0.22	0.12	0.08 (-0.46, -0.03)
Androstenedione ng/ml	-0.08	0.04	0.03 (-0.15, -0.01)
Oestrone pg/ml	-19.77	7.97	0.02 (-35.93, -3.62)

Table 4.5.4b Comparison androgen, oestradiol and oestrone levels by nuclei count categorised by high/low groups (combined central and intermediate nuclei).

Combined central and intermediate nuclei to total muscle fibre ratio			
Hormonal variable	**Mean difference**	**Standard error**	**p value (95% CI)**
11-Ketotestosterone (11-KT) ng/ml	-0.09	0.08	0.26 (-0.25, 0.07)
11-Ketodihydrotestosterone (11-KDHT) (yes/no)	-0.14	0.07	0.04 (-0.27, -0.01)
11-Ketoandreostenedione (11-KA4) ng/ml	-0.35	0.36	0.34 (-1.08, 0.38)
Testosterone ng/ml	-0.05	0.02	0.04 (-0.09, -0.01)
DHEA ng/ml	-0.22	0.12	0.08 (-0.46, 0.28)
Androstenedione ng/ml	-0.05	0.04	0.17 (-0.14,0.02)
Oestrone pg/ml	7.06	12.32	0.57 (-17.91, 32.03)

4.5.5 Relationship between androgen levels to skeletal muscle nuclei count, muscle fibre size and muscle fibre type distribution.

As the relationship between androgens and centralised nuclei count were non-linear, an exponential regression was performed to assess the relationship between androgen levels to central nuclei:fibre ratio. Higher serum testosterone and androstenedione levels were significantly associated with lower levels of central nuclei to muscle fibre ratio (Table 4.5.5a, Figures 4.5.5 a-b).

Table 4.5.5a Exponential regression between testosterone and 11KT to central nuclei: muscle fibre ratio

Variable	r^2	Beta	p value
Testosterone	0.11	-13.89	0.04
Androstenedione	0.11	-14.21	0.04
11-KT	0.08	-13.44	0.08

Figure 4.5.5a: Exponential regression scatter plot central nuclei: muscle fibre ratio to serum testosterone

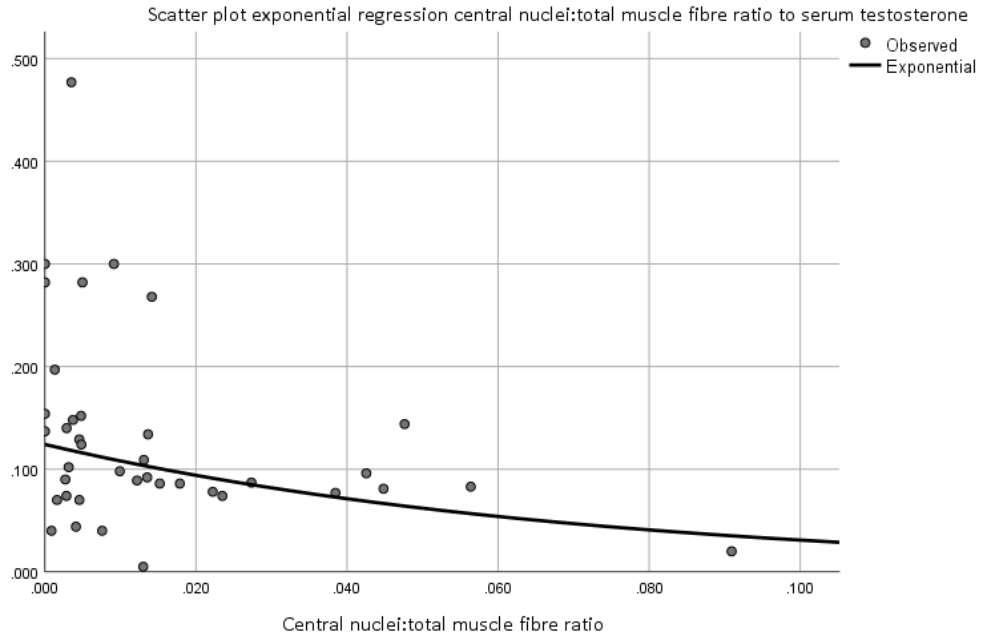
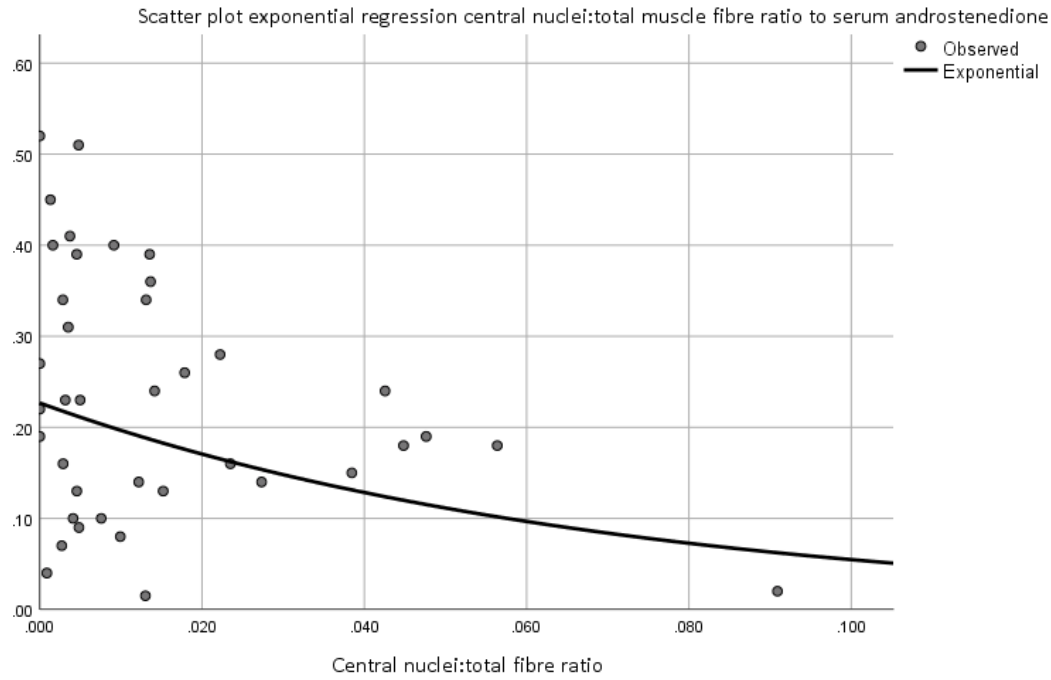


Figure 4.5.5b Exponential regression scatter central nuclei: muscle fibre ratio to androstenedione



In view of the difference in androgen levels between groups the relationship between androgen to muscle fibre area was further assessed in a univariate regression analysis. Higher testosterone levels were associated with larger Type I and Type II muscle fibre size (Table 4.5.5b). Of the androgens accessed, dihydrotestosterone was associated with increased percentage of Type II muscle fibre (Table 4.5.5c).

Table 4.5.5b Univariate regression analysis androgens to muscle fibre cross sectional area

Variable	Muscle fibre cross sectional area µm^2	Beta 95% CI	r^2	p value
11-ketotestosterone	4000-6000	-20.45 (-40.43, -0.47)	0.11	0.05
Testosterone	6001-8000	40.60 (17.19, 80.01)	0.22	<0.01
Testosterone	>8000	17.00 (-0.10, 34.11)	0.10	0.05
Dihydrotestosterone	>8000	85.77 (22.92, 148.63)	0.45	0.01
Testosterone	4000-6000	48.325 (10.01, 86.64)	0.015	0.02
Testosterone	6001-8000	16.75 (0.67, 32.84)	0.042	0.04

Table 4.5.5c Univariate regression analysis androgens to percent Type I and Type II muscle fibre.

Androgen	Muscle fibre parameter	Beta 95% CI	r^2	p value
Dihydrotestosterone	Percent fibre MHC1	-0.001 (-0.002, 0.000)	0.309	0.05
	Percent fibre MHC2	0.001 (0.00,0.002)	0.309	0.05

MCH1: Type I muscle fibre, MHC 2: Type II muscle fibre

4.6 Discussion

This study is an extension of participants from Chapter 3 (Cohort 2), evaluating the sex steroid profile in this group of women, and its relationship to skeletal muscle mass and strength. The relationship between androgens to muscle nuclei, muscle fibre size and muscle fibre distribution were also examined, and a comparison of oestradiol and oestrone performed as a secondary analysis.

The key findings from this study are lowered levels in several androgens observed in the hip fracture group. These are serum testosterone, DHEA and androstenedione. Of the 11-oxygenated steroids, 11-KT and 11- KA were noted to be lowest in the hip fracture group.

11-KT was also observed to have a positive relationship with grip strength, a finding which has not been reported in women with hip fracture before.

DHEA and 11-KT were positively associated with grip strength and higher androgen levels were associated with larger Type I and II muscle fibre. These findings were consistent with previous studies where testosterone use in eugonadal men was associated with an increase in muscle satellite cells, myonuclei [202] and Type I and Type II muscle hypertrophy [203]. However contrary to the study hypothesis, there was an inverse relationship between central muscle nuclei and androgen levels indicating the effects of androgen on muscle regeneration in women differs compared to men. While increase muscle regeneration is associated with higher levels of testosterone in men, observation from this study suggest increased muscle regeneration is a compensatory response to lowered androgen levels in women.

Significantly lower serum gonadotrophins and higher serum oestradiol and oestrone levels were detected in women with hip fracture despite an older age group. These differences were observed following age adjustment, suggesting other factors contribute to this observation. High serum oestradiol levels in the hip fracture group compared to other women were somewhat unexpected. As increased aromatisation have been reported before in critically ill patients [413], a ratio of oestrone to A4 was examined as a crude measure of increased aromatisation activity, and a log transformation performed in view of the non-parametric distribution. Ratios of oestrone:A4 were highest in women with hip fracture but levels in women with hip replacement were comparatively lower than healthy controls.

To discuss this further, it is important to highlight that peripheral blood sampling in these groups were performed in a stressed state (in the hip fracture group), while those in the healthy replacement group and healthy controls had blood samples taken in a non-stressed

state. This may support 2 separate mechanisms to explain the differences observed in oestrone levels in these women:

High oestrone levels in the hip fracture group are a result of acute illness or increase age:

High oestrone levels have been reported in women older than 70 years of age in a large cohort study in Australian women [414]. As most women in the hip fracture group (16/19) are over the age of 70, elevations in oestrone levels may be due to their older age. Alternatively the high oestrone and oestrone:A4 ratios observed suggest increased aromatase activity in acute illness.

Several studies in post-menopausal women have examined the effects of acute illness on gonadotrophins and oestradiol. In intensive care unit patients, there was a reduction in gonadotrophins and up to five-fold increase in oestradiol levels [415]. Elevations in oestrone levels have also been reported in these women [416] and relates to the increase in peripheral aromatization of adrenal androgens to oestrogen [413] during acute illness as reflected by an increase in aromatase mRNA expression. Furthermore these changes have been observed not only in women, but also in men who are critically ill [417, 418]. The concurrent low gonadotrophin levels in these women were also consistent with previous studies and suggest either hypogonadotrophic hypogonadism [419] with acute illness or a negative response to elevated oestrogen/oestrone levels [420]. The significance of high serum oestrogen/oestrone remains uncertain but have been hypothesized to stimulate the immune response, act as a prothrombotic factor [415] and protect against neuronal damage during hypoxia [413].

High oestrone levels in healthy controls compared to hip replacement may suggest oestrone levels have a positive effect on the muscle in a group of women whose blood sample was collected in a non-stressed state:

When comparing women with hip replacement to healthy controls, serum oestrone and oestrone: A4 ratio were significantly higher in healthy controls. The presence of oestrogen receptors have been reported in the muscle before [386, 396, 421]. Estrogen have been demonstrated to have anti-apoptotic effects on the muscle via the AKT pathway [397], promote myoblast growth [400], maintain satellite cell function and muscle regeneration [398, 399].

Clinical studies using estrogen in women in their early post-menopausal years demonstrate a reduction in markers of muscle atrophy [422], while HRT users have stronger muscle strength [403] and contractile function [404]. These findings combined suggest oestrogen has a protective role on the skeletal muscle and may explain the higher levels of oestrone and oestrone: A4 ratio observed in healthy controls in our study.

This finding somewhat contradicts our analysis of increase odds ratio for sarcopenia with increasing oestrone levels in our study. The association between oestrone to sarcopenia status however became non-significant after exclusion of women from the hip fracture group. Hence this would still explain the likelihood of two separate mechanisms observed in changes in serum oestrone levels in these women i.e. high serum oestrone levels and its association with increased odds ratio for sarcopenia in the most sarcopenic group (hip fracture) in an acute setting, and higher serum oestrone levels in healthy controls compared to hip replacement group in a non-acute setting, which may have a positive effect on skeletal muscle.

<u>Study strengths and limitations:</u>

Although limited by the small sample size, this study has demonstrated new findings which have not been reported in the hip fracture population before; the low levels of 11-oxygenated androgens and high oestrone levels in the hip fracture group in an acute illness

state, and the increase in skeletal muscle central nuclei as a response to low androgen levels in these women. These new insights can thus be used to guide future large scale studies to confirm the underlying mechanisms to muscle wasting in these women.

There are however several limitations. Due to the cross sectional nature of the study, inferences can only be made based on association rather than causation. The lack of serial hormonal testing at different time points limits the ability to track the trajectory of changes observed in gonadotrophins and sex steroid levels from the acute phase of illness to recovery. Although testosterone levels may be low in an acute setting, the concurrent low DHEA levels in our study suggest that the changes observed in androgens are likely chronic as one would expect an increase in DHEA levels in the hip fracture group, driven by the acute rise in ACTH levels. Moreover while low androgen levels may be a risk factor for low skeletal muscle mass and strength in these women, the association with long term clinically relevant outcomes remains unanswered due to the nature of the study. Lastly the small sample size does limit the generalisability and power of study findings.

4.7 Conclusion

Several androgen including the 11-oxygenated steroids (testosterone, DHEA, androstenedione, 11-KT and 11-KA) were noted to be reduced in hip fracture and hip replacement group. 11-KT was associated with a reduction in grip strength further reinforcing the link between androgens and muscle. In women, lowered androgens were associated with increase markers of muscle regeneration as reflected by central nuclei count and likely reflects a compensatory response to low androgen levels.

Moving forwards an important aim in future studies is to examine the effect of testosterone supplementation on skeletal muscle particularly at the cellular and molecular level in women, and its long term effects in terms of clinically relevant outcomes. While the focus of this study was not to evaluate the effect of oestrogen in muscle, the higher oestrone levels

observed in healthy controls compared to hip replacement suggest a potential positive effect of oestrogen on skeletal muscle in post-menopausal women. Future studies aimed at examining the role of androgens and its aromatisation to oestrogen would provide a better understanding on the effects of these hormones on the muscle in older women.

In studies undertaken in an acute setting, it is important to perform repeated blood sampling to enable comparison and analysis of the trajectory of these hormonal changes and its association with clinically relevant outcomes. A large scale prospective follow up study in this regard would provide further robustness. Given the positive association observed with androgens to muscle in this study interrogation of the molecular pathway and protein signalling would be helpful. This will be attempted in Chapter 5 with western blot analysis and histology assessment.

Acknowledgements:

1. Reena Desai and Professor David Handelsman at ANZAC Research Institute for the processing of serum samples and provision of LCMS methods for reporting in this thesis
2. Professor Karl Storbeck for intellectual input on testing of 11-OXO androgens
3. Mr Sachin Nair for assistance with muscle nuclei count

Chapter 5: Exploration of protein signalling in skeletal muscle regulation by western blot and histology analysis.

5.1 Abstract

Findings from Chapter 3 support the observation that older women with hip fracture are a group of sarcopenic women based on low skeletal muscle mass and grip strength, compared to women awaiting hip replacement and healthy controls. Characterisation of hormonal profile in these women suggest low androgen levels (testosterone and several 11-OXO androgens), IGF-1, IGFBP-3 and beta cell function contribute towards the increased risk of muscle atrophy in these women. In this study, the activity of several intracellular signalling pathways involved in skeletal muscle regulation were examined and its association to these hormones further analysed.

Pertinent findings from this chapter indicate a lack of activation in the mTOR pathway, while MURF-1 appears to be consistently associated with low skeletal muscle mass and strength supporting its role as a key pathway in muscle atrophy in these women. Increased SMAD 3 protein levels points towards increased activity of the TGF-β pathway providing additional insight into another pathway to muscle atrophy in women with hip fracture. On the contrary SMAD 7 levels were unexpectedly higher in women with hip fracture, suggesting a likely compensatory response to counteract the elevation in SMAD 3 levels. Combined these findings suggest these pathways could be a focus for future studies particularly targeting their interactions with androgens, beta cell function, IGF-1 and IGFBP-3.

5.2 Introduction

Muscle regulation is a complex interplay between anabolic and catabolic pathways. Moreover, these interactions are influenced by the presence of several regulatory hormones and external factors such as impaired mobility, physical activity, and nutrition. A disparity in the balance between these different factors ultimately results in reduced muscle

protein synthesis and an increase in muscle atrophy with the subsequent outcome of impaired muscle function [423].

In chapters 3 and 4, hormones implicated in muscle regulation and its association with measures of skeletal muscle mass, strength, and central nuclei (as a marker of skeletal muscle regeneration) were examined. Androgens, insulin-like growth factor-1 (IGF-1), Insulin like Growth Factor Binding Protein 3 (IGFBP3) and insulin (beta cell function by HOMA Beta) were lower in women with hip fracture compared to women awaiting hip replacement and healthy controls. This chapter further explores the relationship between these hormones of interest to the intracellular signalling proteins involved in muscle regulation by western blot analysis.

As muscle quality is impacted by the presence of skeletal muscle adiposity and fibrosis [424], histological analysis was performed looking for evidence of skeletal muscle fibrosis, and adiposity was assessed by CT thigh Hounsfield units. Increased age was also associated with changes in muscle fibre type and size [161], thus muscle samples were analysed to determine the distribution of Type I and Type II muscle fibres and their cross-sectional area in these women.

The different pathways involved in muscle homeostasis described in Chapter 1 are briefly outlined for convenience in the following diagrams. Signalling proteins from these pathways will be targets of interest on western blot analysis. The IGF-1, IGFBP-3, insulin (HOMA Beta) and its link to the mechanistic target of rapamycin (mTOR) pathways are represented by mTOR, protein kinase B (AKT), ribosomal S6 (RS6) and 4E binding protein-1 (4EBP1). Note that 4EBP1 in its non-phosphorylated form are bound to eukaryotic translation initiation factor 4E (eIF4E). As the role of eIF4E is to associate with eukaryotic translation initiation factor 4G (eIF4G) to initiate protein translation, 4EBP1 limits this process by sequestration of eIF4E (Figure 5.2a).

The atrophy (catabolic) pathway is represented by the muscle ring finger-1 (MURF-1) (Figure 5.2a). The TGFβ pathway is represented by SMAD 3 and SMAD 7 (Figure 5.2b) while the androgen pathway is represented by the androgen receptor (Figure 5.2c).

Figure 5.2a: Signalling proteins in involved in the mTOR pathway.
Highlighted boxes for signalling proteins: Blue indicate stimulatory, pink indicate atrophic pathways
Black arrows indicate stimulatory while red arrows indicate inhibitory effect.

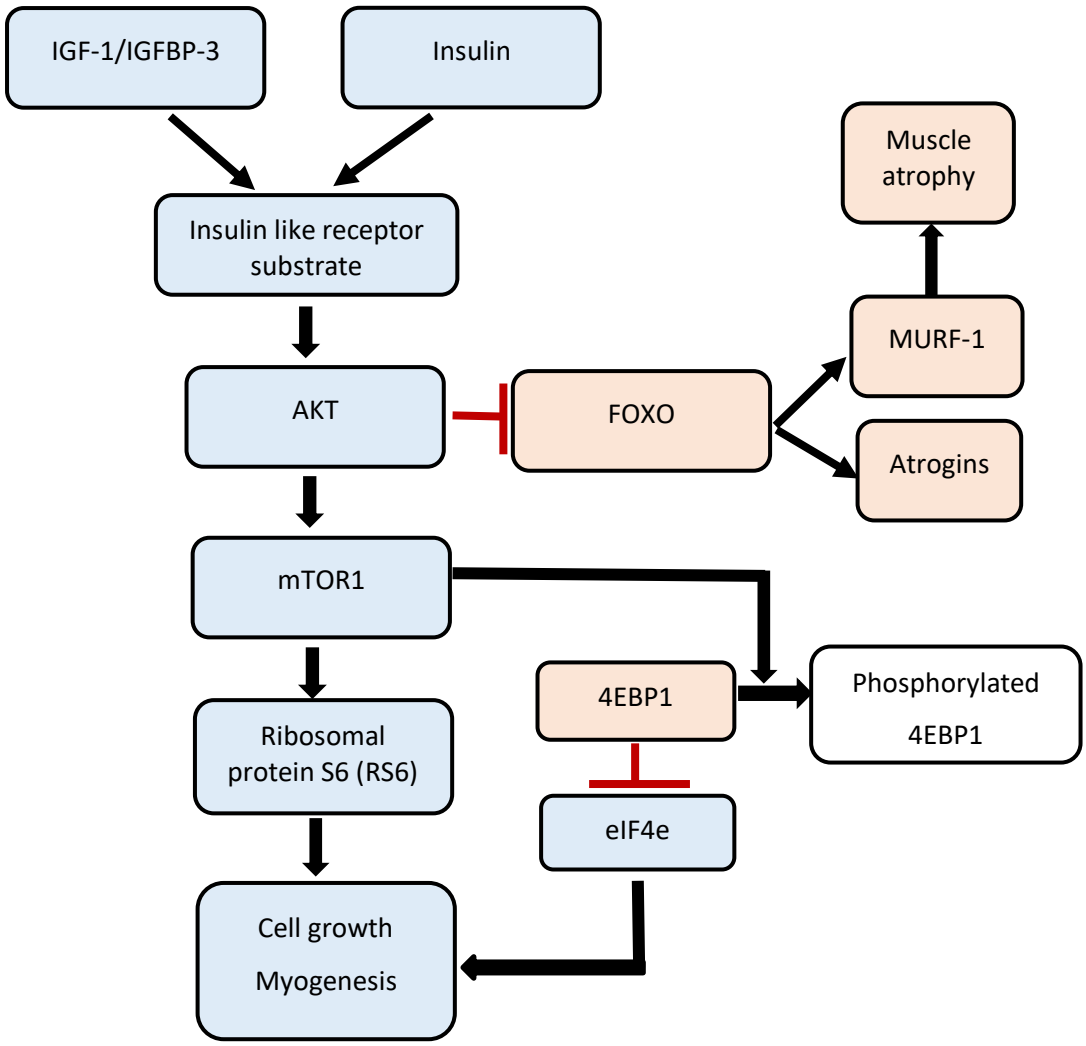


Figure 5.2b: Signalling proteins involved in the TGF-β pathway [270].
Highlighted boxes for signalling proteins: Blue indicate stimulatory, pink indicate atrophic pathways
Black arrows indicate stimulatory while red arrows indicate inhibitory effect.

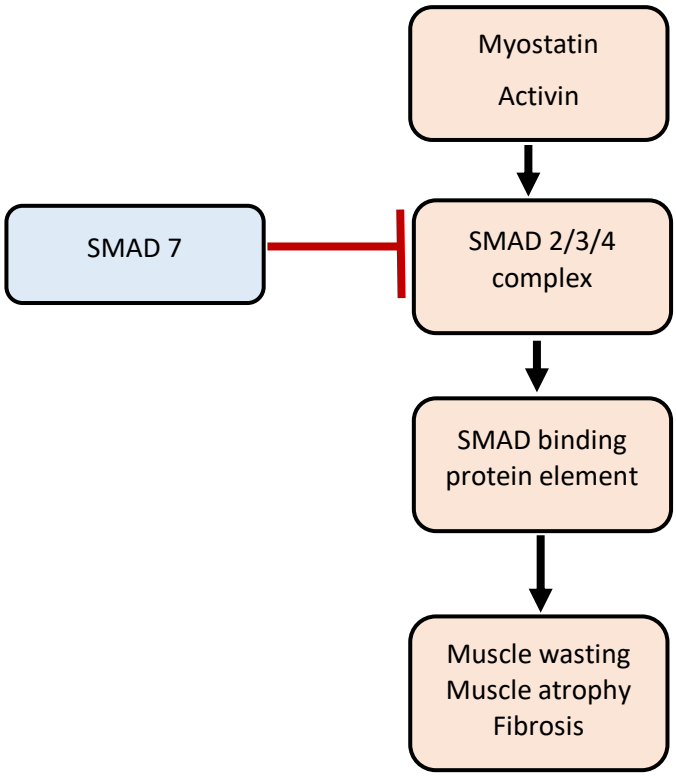
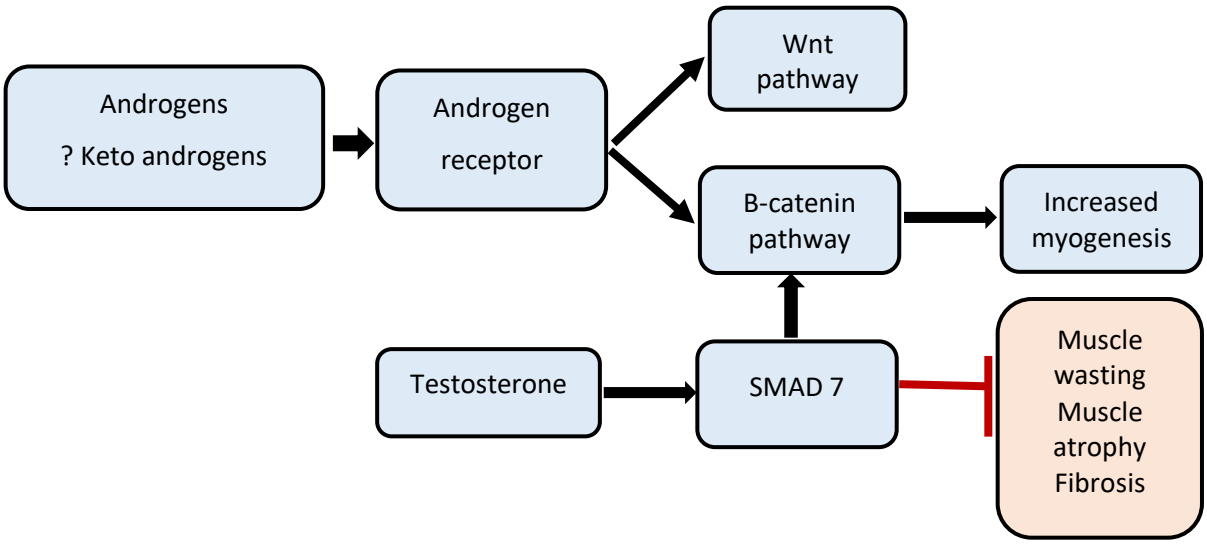


Figure 5.2c: Androgen receptor pathway [206, 425]
Highlighted boxes for signalling proteins: Blue indicate stimulatory, pink indicate atrophic pathways
Black arrows indicate stimulatory while red arrows indicate inhibitory effect.



A summary of hypothesized changes in signalling proteins are listed in Table 5.2a.

Table 5.2a Hypothesized changes in signalling proteins in the sarcopenic group (hip fracture and hip replacement) in comparison to the non-sarcopenic group based on existing literature.

Signalling proteins	**Sarcopenic Group** Hip fracture Hip replacement	**Non sarcopenic Group** Healthy control
mTOR pathway		
mTOR/pmTOR	↓/↓	↑/↑
AKT/pAKT	↓/↓	↑/↑
RibS6/pRibS6	↓	↑
t 4EBP1	↑	↓
p4EBP1	↓	↑
Androgen, TGF-β pathway		
tSMAD 3/pSMAD 3	↑/↑	↓/↓
SMAD 7	↓	↑
Androgen receptor	↓	↑
Atrophy pathway		
MURF-1	↑	↓

mTOR: Mechanistic Target of Rapamycin, pmTOR: phosphorylated mTOR, AKT: Protein Kinase B, pAKT:phosphorylated AKT, pRIBs6: phosphorylated Ribosomal S6,t 4EBP1:Total 4EBP1, p4EBP1: phosphorylated 4EBP1, tSMAD3: total SMAD 3, pSMAD3: phosphorylated SMAD 3.

5.3 Aims

- To explore and compare the signalling proteins in different pathways involved in skeletal muscle regulation by western blot analysis in women with hip fracture compared to women awaiting total hip replacement and healthy controls

- To examine the relationship between signalling proteins to skeletal muscle mass, strength and sarcopenia status in this population

- To examine the relationship between signalling proteins to hormonal levels (androgens, IGF-1, IGFBP3 and insulin by HOMA Beta) in this population

- To measure and compare muscle fibrosis between groups in this population

- To identify and compare Type I and Type II skeletal muscle fibre parameters between groups in this population

- To examine the relationship between Type I and Type II muscle fibre parameters to skeletal muscle mass, strength and sarcopenia status in this population

- To examine the relationship between Type I and Type II muscle fibre parameters to hormonal levels (androgens, IGF-1, IGFBP3 and insulin by HOMA Beta) in this population

5.4 Methods

Participants who were enrolled from Chapter 3 (Cohort 2) were consented for participation in this study. Collection of peripheral blood sampling, muscle biopsy and processing have previously been outlined. Reporting of methods will focus on protein extraction and western blot analysis.

All protein extraction was performed by myself using a protocol provided by Dr Kate Sadler. 65% of the western blot were performed by Dr Evelyn Zacharewicz, and the remainder 35% by myself. Detailed step by step protein extraction and western blot methods are provided in Appendix 5.8.

Protein extraction and western blotting from muscle biopsy samples:

Briefly muscle samples were chipped into 25-30mg sections, homogenised in radioimmunoprecipitation assay (RIPA) buffer (Millipore, Billerica, MD, USA) containing a protease inhibitor and phosphatase inhibitor cocktail (Sigma-Aldrich, Sydney, Australia). Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL). The protein was stored at −80 °C until further use. Western blotting was performed as described in Van Langenberg [426].

Muscle homogenates (20µg) were separated on 4%–15% Criterion TGX Stain-Free precast gels (BioRad, Australia) in Tris/Glycine/SDS buffer solution (Bio-Rad). Proteins samples were transferred for 30 minutes onto Immobilin-FL PVDF membranes (Millipore, Billerica, MA, USA) and membranes scanned to quantify total protein transferred using a Bio-Rad Gel Doc XR+ (Bio-Rad). Membranes were then blocked for 1 h with 5% skim milk powder/10% Tris-buffered saline with 0.1% Tween 20 (TBST) at room temperature. After blocking, membranes were incubated overnight at 4°C in the antibodies outlined in the table below (Table 5.4a).

Table 5.4a: Western blot target antibodies and their manufacturers.

Target antibodies	**Manufacturer (cat number)**	**Dilution**	**Antibody type**
Phosph-mTOR (Ser2448)	Cell Signalling (5536S)	1:1000	Rabbit
Total mTOR	Cell Signalling (4517S)	1:1000	Mouse
Phospho-Akt (Ser473)	Cell Signalling (4060S)	1:2000	Rabbit
Total Akt	Cell Signalling (2920S)	1:2000	Mouse
Phospho- S6 ribosomal protein (Ser235/236)	Cell Signalling (4517S)	1:2000	Rabbit
Total Ribosomal S6	Cell Signalling (2317S)	1:1000	Mouse
Phospho- 4E Binding Protein 1 (4EBP1) (Thr37/46) (236B4)	Cell Signalling (2855S)	1:1000	Rabbit
Total 4E Binding Protein 1 (4EBP-1)	Cell Signalling (9452S)	1:1000	Rabbit
Androgen receptor	Cell Signalling (5153S)	1:2000	Rabbit
Phosphorylated SMAD 3 (S423/S424)	Abcam (ab52903)	1:1000	Rabbit
Total SMAD 3	Cell Signalling (9523S)	1:1000	Rabbit
SMAD 7	Novus (NBP224710)	1:500	Rabbit
Alpha actinin	Sigma (A7732)	1:1000	Mouse
MURF-1 (Muscle Ring Finger 1)	ECM Biosciences (MP3401)	1:1000	Rabbit

The following day, membranes were incubated for 1 hour with fluorescent secondary antibodies (Anti-Mouse IgG (H+L) DylightTM 680 Conjugate or Anti-Rabbit IgG (H+L) DylightTM 800 Conjugate; Cell Signalling Technologies®, Danvers, MA, USA). Membranes were exposed on an Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) and individual protein band optical densities were determined using Image Studio Lite software (V5.2.5; LI-COR Biosciences, Lincoln, Nebraska, USA).

Histology analysis: Picro Sirius red staining for muscle fibrosis:

Picro Sirius Red Staining was performed using a commercially available kit following manufacturer's instructions (Abcam, Melbourne, cat number ab150681). Images of the prepared slides were captured on the E2000 light microscope (Nikon, NSW, AUS). To analyze

for fibrosis, the area of connective tissue (collagen, stained red) and muscle tissue (stained yellow) were quantified using colour thresholding (Olympus CellSens software (Olympus, Australia). The same threshold values were used for all images.

Histology analysis for muscle fibre typing:

Haematoxylin and eosin staining, and immunohistochemistry were previously reported in Chapter 4. Measures of muscle fibre diameter are reported as minimum ferret's diameter (μm) and muscle fibre cross sectional area in μm^2. Minimal ferret's diameter is a measure of 'the closest possible distance between two parallel tangents of a muscle fibre'. It is the preferred method of measurement as it has been shown to have the lowest variant of coefficient and is independent of the angle of the plane of section [427]. Percentage of muscle fibre type were calculated as a proportion of total muscle fibre observed on analysis.

Statistical analysis and reporting of results:

Reporting of western blot analysis results were based on fold change values where mean levels of signalling proteins were normalized to mean levels to healthy controls, presented as mean ± standard deviation. Parametric data are reported as mean ± standard deviation, and non-parametric data are reported as median (interquartile range). ANOVA was used to compare means between the three groups of women, and student's t-Test for comparison of means between two groups of interest (hip fracture to healthy controls, hip fracture to hip replacement and hip replacement to healthy controls). As the groups differed by age, differences observed in muscle fibre was corrected for age as a confounder with ANCOVA analysis.

Univariate linear regression analysis was performed assessing the relationships between the following variables:

- Signalling proteins on western blot analysis to measures of skeletal muscle mass, strength, and sarcopenia status by EWGSOP 1 criteria

- Signalling proteins on western blot analysis to Androgens, IGF-1, IGFBP3 and insulin (HOMA Beta)

- Type I and Type II muscle fibre diameter, cross sectional area and proportion of muscle fibre types to measures of skeletal muscle mass, strength, and sarcopenia status by EWGSOP 1 criteria

- Type I and Type II muscle fibre diameter, cross sectional area and proportion of muscle fibre types to Androgens, IGF-1, IGFBP3 and insulin (HOMA Beta)

Graphs were created using Graph Pad Prism Software Version 8.4.2. Error bars on the graphs are reported as standard error of mean (SEM). Statistical analysis was performed using SPSS version 26 (IBM, Chicago IL, USA). A p value of < 0.05 were used to determine statistical significance in all analysis.

5.5 Results

5.5.1 Demographics:

Table 5.5.1a is a repetition of demographics already previously reported. It is repeated here for convenience.

Table 5.5.1a Demographics of study participants

Category Mean ± SD Median (IQR)	**Hip fracture** (n=20)	**Hip replacement** (n=10)	**Healthy controls** (n=19)	**p value**
Age (Years)	80.7 ± 11.3	72.3 ± 6.0	74.8 ± 8.4	<0.05
Weight (kilograms)	62.3 (57.9,77.3)	76.7 (74.0, 90.0)	66.6 (61.7,71.6)	0.02
Body mass index (kg/m^2)	25.2 (23.1,31.0)	28.1 (26.3, 32.4)	25.6 (23.6,29.1)	0.31
Medication amount	6 (3,10)	7 (4,8)	2 (1,4)	<0.01
Clinical Frailty Score [a]	3 (2, 5)	3 (2, 3)	2 (1, 2)	<0.01

[a] Clinical frailty scale score: 1-Very fit, 2-Well, 3-Managing well, 4-Vulnerable, 5-Mildly frail, 6-Moderately frail, 7-Severely frail, 8-Very Severely frail, 9-Terminally ill.

5.5.2 Groupwise comparison for western blot analysis.

Tables 5.5.2a-c summarizes the groupwise comparison by ANOVA for signalling proteins involved in skeletal muscle regulation. Mean levels for each group are reported as fold change (mean levels from each group normalized to mean levels from the healthy control group).

Mechanistic Target of Rapamycin (mTOR) pathway

In the IGF-mTOR pathway, there was no significant difference observed in mechanistic target of rapamycin (mTOR), protein kinase B (AKT) and ribosomal S6 (RS6) levels between groups. Total 4EBP1 were significantly higher while the ratio of phosphorylated to total 4EBP1 were lower in the hip fracture group compared to healthy controls. (Table 5.5.2a, Figure 5.5.2a). Women in the hip replacement group had comparatively higher total 4EBP1 and lower phosphorylated to total 4EBP1 ratio when compared to healthy controls.

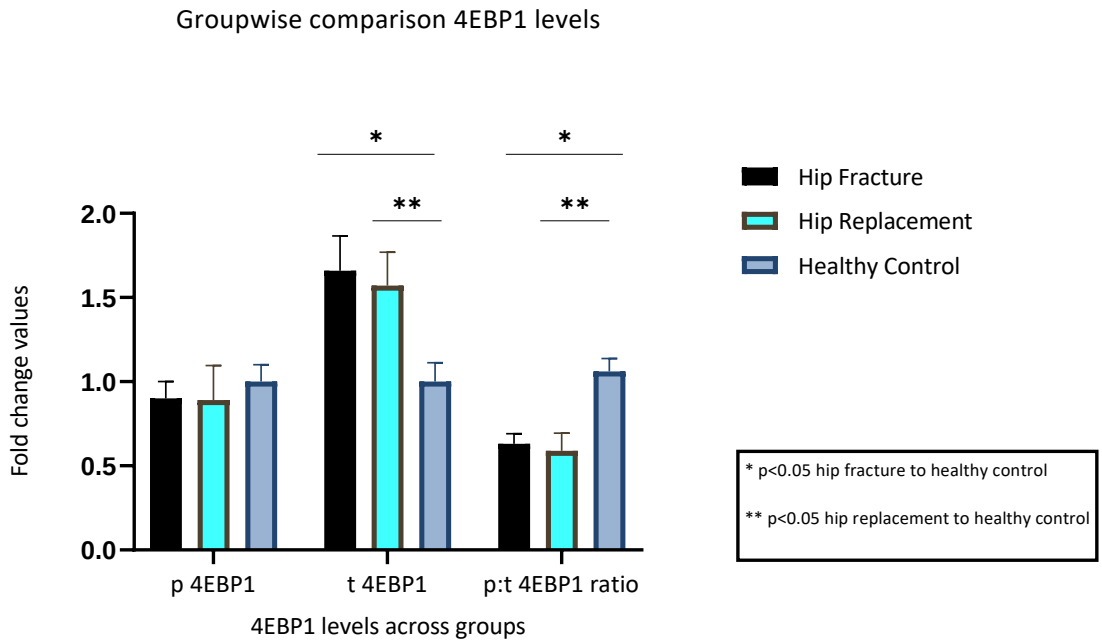
Table 5.5.2a Comparison of signalling proteins in mechanistic target of rapamycin (mTOR) pathways between groups

Signalling proteins Expressed as fold change Mean ± SD	**Hip fracture** (n=20)	**Hip replacement** (n=10)	**Healthy control** (n=14)	**p value**
Mechanistic Target of Rapamycin (mTOR) pathway				
Phosphorylated mTOR (pmTOR)	1.01±0.42	0.72 ±0.56	1.00±0.45	0.24
Total mTOR (tmTOR)	1.10±0.25	1.03±0.42	1.00±0.20	0.62
pmTOR : tmTOR	0.95±0.36	0.71±0.46	1.00±0.32	0.16
Phosphorylated AKT (pAKT)	1.13±1.32	0.99±2.16	1.00±0.61	0.95
Total AKT (tAKT)	1.08±0.45	0.88±0.42	1.00±0.21	0.43
pAKT: tAKT	0.93±0.66	0.86±1.44	1.00±0.58	0.92
Phosphorylated RibS6 (pRS6)	3.66±9.57	1.52±2.97	1.00±1.26	0.48
Total Rib S6 (tRS6)	1.52±1.09	1.30±0.49	1.00±0.27	0.18
pRS6: tRS6	1.59±2.28	0.91±1.66	0.89±1.06	0.47
Phosphorylated 4EBP1 (p4EBP1)	0.90±0.45	0.89±0.65	1.00±0.38	0.80
Total 4EBP1 (t4EBP1)	1.66±0.92 [a]	1.57±0.63 [b]	1.00±0.42 [a b]	0.04
p4EBP1: t4EBP1	0.63±0.27 [a]	0.59±0.33 [b]	1.06±0.29 [a b]	<0.01

[a] p value < 0.05: t-test comparison hip fracture to healthy controls

[b] p value <0.05: t-test comparison hip replacement to healthy controls

Figure 5.5.2a Comparison 4EBP1 levels between groups. Error bars are reported as standard error of mean (SEM).



<u>TGF-β, androgen receptor pathway</u>

In the TFGF-β pathway phosphorylated SMAD 3 and total SMAD3 levels were significantly higher in women with hip fracture compared to healthy controls ($p<0.05$). Ratios of phosphorylated to total SMAD 3 levels were significantly lower in women with hip fracture and hip replacement compared to healthy controls (Table 5.5.2b, Figure 5.5.2b).

Women with hip fracture and hip replacement had significantly higher SMAD 7 levels compared to healthy controls ($p<0.01$), resulting in lower phosphorylated SMAD3 to SMAD 7 ratios ($p<0.01$) in these women (Table 5.5.2b, Figures 5.5.2c-d).

Table 5.5.2b Comparison of signalling proteins in androgen receptor and TGF-β pathways between groups

Signalling proteins Expressed as fold change Mean ± SD	**Hip fracture** (n=20)	**Hip replacement** (n=10)	**Healthy controls** (n=14)	p value
Androgen receptor, TGF-β Pathway				
Androgen receptor	0.93±0.49	0.87±0.38	1.00±0.33	0.75
Phosphorylated SMAD 3 (pSMAD3)	1.61±0.86 [a]	1.12±0.76	1.00±0.56 [a]	0.05
Total SMAD 3 (tSMAD3)	7.23±12.82 [a]	4.67±2.05 [b]	1.00±1.57 [ab]	0.14
pSMAD3: tSMAD3	0.82±1.13 [a]	0.27±0.18 [b]	2.80±2.35 [ab]	<0.01
SMAD 7	6.15±4.51 [a]	6.14±2.89 [b]	1.00±0.95 [ab]	<0.01
pSMAD3: SMAD 7	0.50±0.66 [a]	0.19±0.11 [b]	2.39±2.98 [ab]	<0.01
tSMAD3: SMAD 7	1.03±1.10	0.85±0.49	0.50±0.13	0.85

[a] p value < 0.05: t-test comparison hip fracture to healthy controls

[b] p value <0.05: t-test comparison hip replacement to healthy controls

Figure 5.5.2b Comparison SMAD3 levels between groups. Error bars are reported as SEM

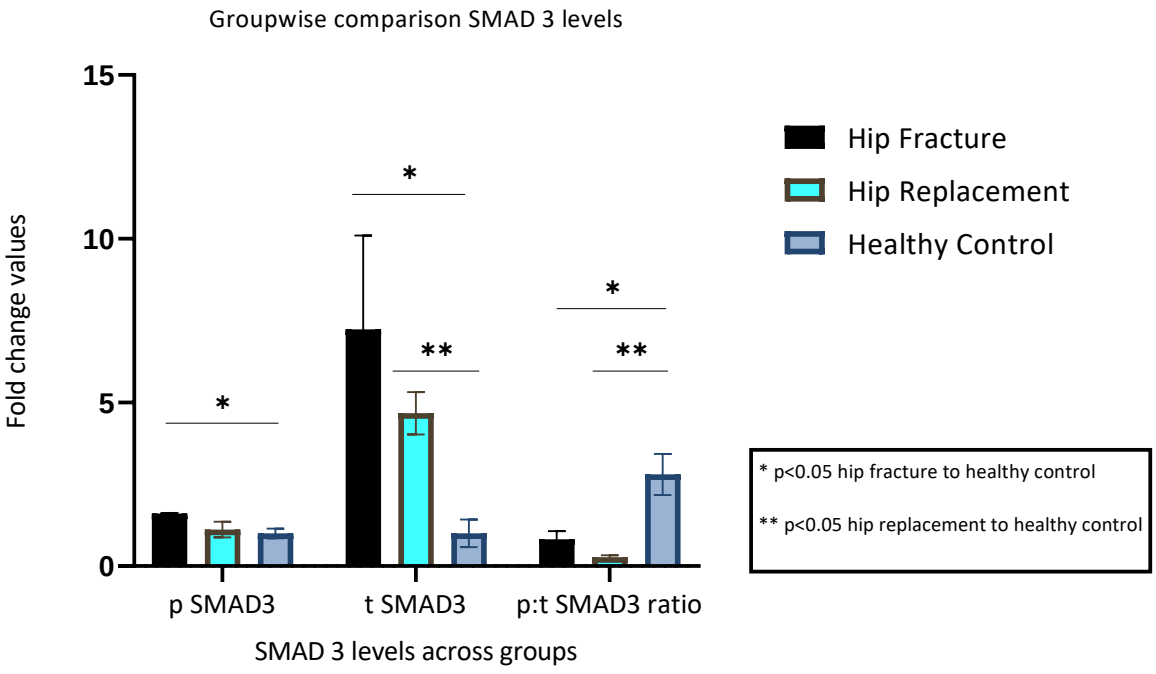
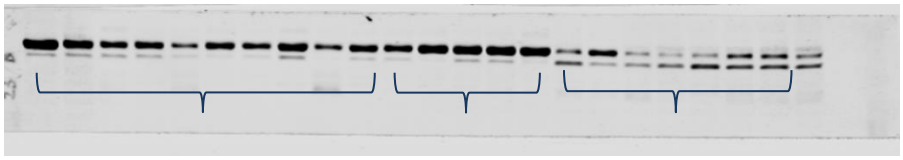


Figure 5.5.2c Western blot results and graph showing SMAD 7 comparison between groups. Error bars are reported as SEM.



HF: Hip fracture, HR: Hip replacement, HC: Healthy control

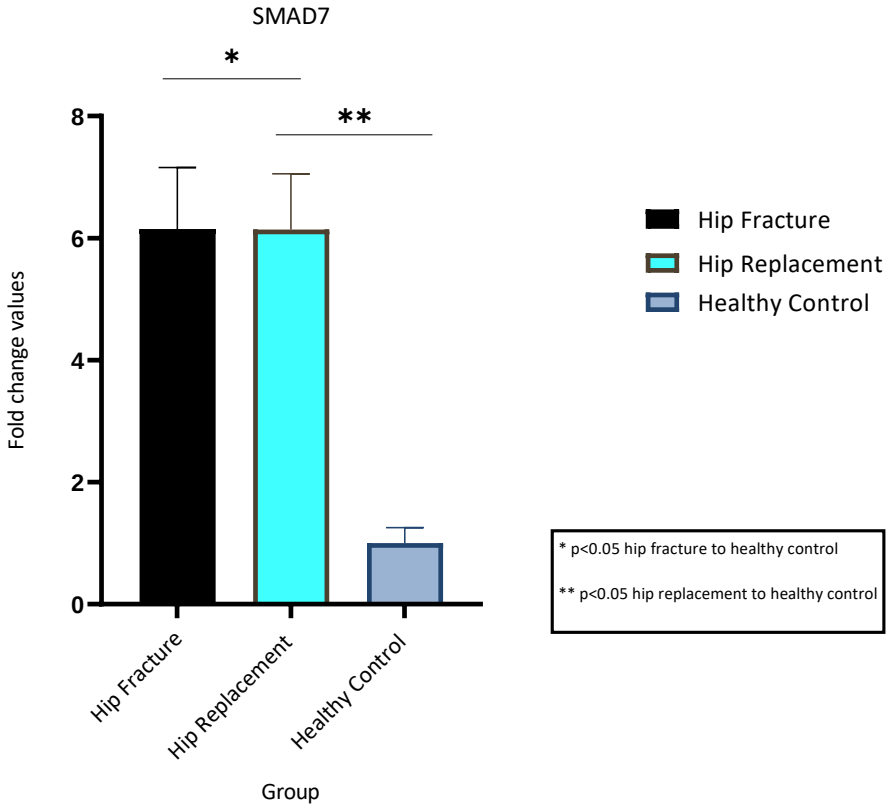
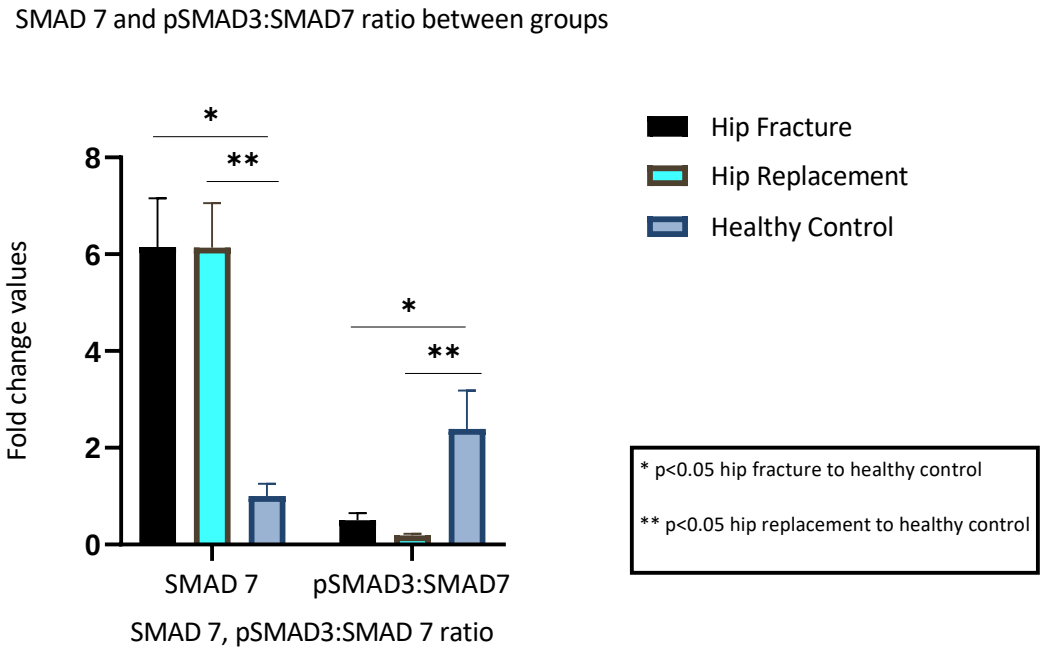


Figure 5.5.2d Comparison SMAD7, and phosphorylated SMAD 3 to SMAD 7 levels between groups. Error bars are reported as SEM



MURF-1 pathway

In the atrophy pathway, MURF-1 levels were significantly higher in women with hip fracture compared to healthy controls (p=0.01). (Table 5.5.2c).

Table 5.5.2c Comparison of MURF-1 signalling protein between groups

Signalling proteins Expressed as fold change Mean ± SD	**Hip fracture** (n=20)	**Hip replacement** (n=10)	**Healthy controls** (n=14)	p value
Muscle Ring Finger-1 (MURF1)	1.98±1.54 [a]	1.28±0.74	1.00±0.44 [a]	0.05

[a] p value < 0.05: t-test comparison hip fracture to healthy controls

5.5.3 Univariate regression analysis between signalling proteins on western blot to measures of skeletal muscle mass, strength, and sarcopenia status

Univariate regression analysis was performed assessing the relationship between signalling proteins to measures of skeletal muscle mass and strength (Tables 5.5.3a-d).

Signalling proteins to muscle mass (muscle area) by CT:

Low muscle area by CT was associated with high levels of phosphorylated SMAD 3 (β -9.42, p =0.021) and MURF-1 (β-4.77, p=0.056) (Table 5.5.3a).

Table 5.5.3a Univariate regression analysis between targets on western blot analysis to muscle area on CT

Muscle area on CT mid-thigh from one leg (cm^2)			
Category	**r^2**	**Beta (95% CI)**	**p value**
Phosphorylated SMAD 3 (pSMAD3)	0.122	-9.42 (-17.38, -1.46)	0.02
MURF1	0.086	-4.77 (-9.67,0.12)	0.06

Signalling proteins to muscle mass by DEXA and BIA:

Except for MURF-1, there was no significant relationship observed between signalling proteins to measures of skeletal muscle mass by DEXA and BIA.

High MURF-1 levels was associated with low muscle mass by DEXA (β -1.82, p=0.038) and dry lean mass by bioelectrical impedance analysis (β -1.15, p=0.003) (Tables 5.5.3b-c).

Table 5.5.3b Univariate regression analysis between MURF-1 to DEXA skeletal muscle mass

DEXA skeletal muscle mass (kg)			
Category	**r^2**	**Beta (95% CI)**	**p value**
MURF1	0.124	-1.82 (-3.53, -0.10)	0.04

Table 5.5.3c Univariate regression analysis between MURF-1 to skeletal muscle mass (dry lean mass and absolute skeletal muscle mass) on BIA

Dry lean mass by bioelectrical impedance analysis (kg)			
Category	**r^2**	**Beta (95% CI)**	**p value**
MURF-1	0.192	-1.15 (-1.89, -0.42)	<0.01
Skeletal muscle mass by calculated by Janssen regression equation using resistance from BIA (kg)			
Category	**r^2**	**Beta (95% CI)**	**p value**
MURF-1	0.082	-0.85 (-1.72, 0.04)	0.06

Signalling proteins to muscle function by grip strength:

High androgen receptor levels was associated with increased grip strength (β 6.59, p=0.02), while MURF-1 was inversely related to grip strength (Table 5.5.3d).

Table 5.5.3d Univariate regression analysis between targets on western blot analysis to muscle function by grip strength

Grip strength (kg)			
Category	**r^2**	**Beta (95% CI)**	**p value**
Androgen receptor	0.124	6.59 (1.13, 12.04)	0.02
MURF-1	0.120	-2.26 (-4.16, -0.35)	0.02

The relationship between signalling proteins to sarcopenia status (yes/no) based on the EWSGOP1 criteria was assessed in a binary logistic regression analysis. MURF-1 was the only protein which were significantly related to sarcopenia status. High MURF-1 levels was associated with increased likelihood of being sarcopenic. (Odds ratio: 1.94, p=0.04). For every unit increase in MURF-1 fold change levels, the odds of being sarcopenic increases by a factor of 1.94. (Table 5.5.3e).

Table 5.5.3e Binary logistic regression analysis to sarcopenia status

Binary logistic regression to sarcopenia status by EWGSOP1 criteria				
Category	**r^2**	**Beta (95% CI)**	**Odds ratio**	**p value**
MURF-1	0.113	0.660 (1.03, 3.64)	1.94	0.04

5.5.4 Univariate regression analysis between androgens, IGF-1, IGFBP3 and insulin to signalling proteins on western blot analysis

The relationship between androgens, IGF-1, IGFBP3 and insulin (HOMA Beta) to signalling protein on western blot analysis was assessed in a univariate regression analysis, with signalling proteins as the dependent variable (Table 5.5.4a).

mTOR pathway:

There was a positive relationship between IGF-1 and IGFBP-3 to phosphorylated to total 4EBP1 ratio, while IGFBP3 was associated with lower total 4EBP1 levels.

TGF-β pathway:

Free testosterone and dehydroepiandrosterone (DHEA) was negatively associated with phosphorylated SMAD3, while high SMAD 7 levels was associated with low free testosterone, DHEA, 11-ketotestosterone and 11-ketoandrostenedione levels.

MURF-1 pathway:

Low MURF-1 levels were associated with high serum IGF-1 and insulin levels.

Table 5.5.4a Univariate linear regression analysis between hormonal levels to signalling protein on western blot analysis

Hormonal levels (Independent variable)	**Protein signalling targets** (Dependent variable)	**r^2**	**Beta (95% CI)**	**p value**
mTOR pathway				
IGFBP3	t4EBP1	0.145	-0.008 (-0.014, -0.002)	0.01
IGFBP3	p4EBP1: t4EBP1	0.114	0.003 (0.000, 0.006)	0.03
IGF-1		0.094	0.017 (0.000,0.034)	0.05
Androgen receptor, TGF-β Pathway				
Free testosterone	pSMAD 3	0.114	-0.072 (-0.14, -0.01)	0.03
DHEA		0.108	-0.486 (-0.93, -0.05)	0.03
Free testosterone	SMAD7	0.147	-0.469 (-0.83, -0.11)	0.01
11-Ketotestosterone		0.163	-7.93 (-13.61, -2.25)	<0.01
11-Ketoandrostenedione		0.126	-1.59 (-2.90, -0.27)	0.02
DHEA		0.111	-2.81 (-5.32, -0.30)	0.03
Atrophy pathway				
IGF-1	MURF-1	0.097	-0.06 (-0.115, -0.003)	0.04
Insulin		0.133	-0.098 (-0.177, -0.020)	0.02
HOMA Beta		0.076	-0.015 (-0.031, 0.001)	0.07

IGF-1: Insulin like Growth Factor-1, IGFBP-3: Insulin like Growth Factor Binding Protein-3, DHEA: Dehydroepiandrosterone, p:phosphorylated, t:total

5.5.5 Histology analysis for fibrosis

TGF-β is known to be a driver of fibrosis in tissues. As there was a difference observed in signalling proteins in the TGF-β pathway and muscle quality is affected by fibrosis, histology analysis was performed looking for evidence of muscle fibrosis. Tissue samples available for analysis were from 18/20 (hip fracture), 10/10 (hip replacement) and 13/19 (healthy control) women in the whole group. This is reported in Table 5.5.5a, Figure 5.5.5a.

Women in the hip fracture and hip replacement group were observed to have a higher degree of muscle fibrosis than healthy controls but these differences were not statistically significant. This likely relates to the wide variance observed in women with hip fracture and hip replacement groups.

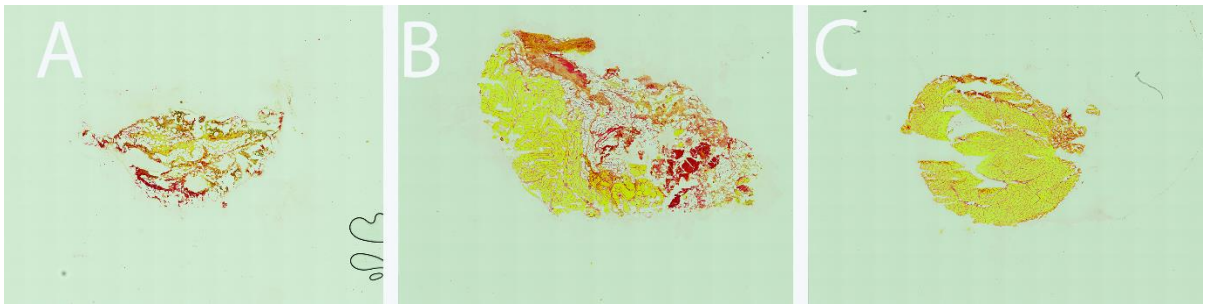
Table 5.5.5a Groupwise comparison for degree of fibrosis and percentage between groups

Category Mean ± SD	**Hip fracture** (n=18)	**Hip replacement** (n=10)	**Healthy controls** (n=13)	**p value** All groups (ANOVA)	**p value** HF: HC (t-test)	**p value** HR: HC (t test)
Percentage muscle fibrosis (%)	11.78 ± 17.2	13.89 ± 16.2	4.93 ± 3.4	0.27	0.12	0.12

HF:HC: Comparison hip fracture to healthy control, HR:HC: Comparison hip replacement to healthy control

To determine if there was a relationship between hormonal levels to percentage of fibrosis observed, a univariate regression analysis was performed between hormonal levels and fibrosis percentage. Increased serum free testosterone was associated with lower percentage of muscle fibrosis [β=-1.53, r^2=0.139, p=0.02 (95%CI -2.82, -0.25)].

Figure 5.5.5a: Histology images representative of each group (A: Hip fracture, B: Hip replacement, C: Healthy Control). Areas staining red are indicative of the presence of collagen and fibrosis while yellow indicates the presence of muscle fibres. Graph below images showing groupwise comparison for percentage area fibrosis. Error bars are reported as SEM.



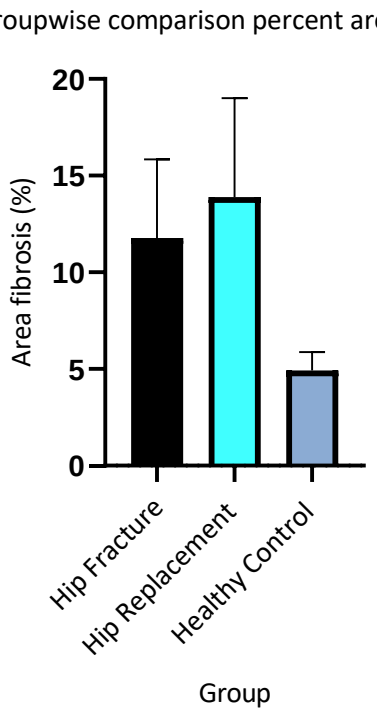
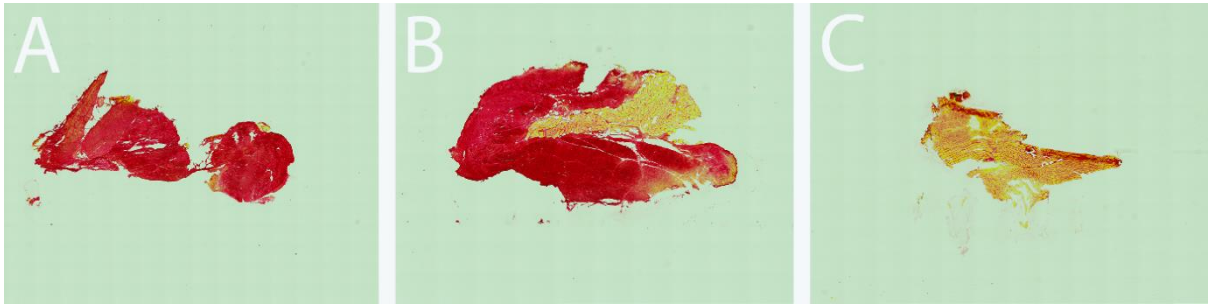


Figure 5.5.5b shows histology slides from individuals from each group (hip fracture, hip replacement, and healthy control) with the highest percentage of fibrosis with age and grip strength listed. Areas staining red are indicative of the presence of collagen and fibrosis while yellow indicates the presence of muscle fibres. Neither increased age nor low grip strength appears to be a common factor identified to explain the degrees of fibrosis observed.

Figure 5.5.5b: Histology slides from three individuals from each group the with highest level of fibrosis.



A: Hip Fracture	B: Hip replacement	C: Healthy control
Sum area fibrosis: 86%	Sum area fibrosis: 71%	Sum area fibrosis: 15%
Age: 63	Age: 82	Age: 71
Grip strength: 22kg	Grip strength :13kg	Grip strength: 22.6kg

5.5.6 Comparison of muscle fibre parameters between groups

Muscle fibre diameter and cross-sectional area between groups

A comparison of skeletal muscle fibre types was performed between the three groups. (Table 5.5.6a, Figure 5.5.6a-b). Minimal ferret's diameter [427], a preferred measure of muscle fibre diameter shows no significant difference between groups. Women in the healthy control group were observed to have larger Type I and Type II muscle fibre diameter compared to the other groups but failed to reach statistical significance (p= 0.16, 0.13).

A similar trend was also observed in muscle cross-sectional area, women from the healthy control group were observed to have larger muscle fibre cross-sectional area but was not statistically significant (p=0.16, 0.08) (Table 5.5.6a, Figure 5.5.6b).

Student's T test comparison between women with hip fracture to hip replacement, hip fracture to healthy control, and hip replacement to healthy control did not detect any significant difference between groups.

Table 5.5.6a: Groupwise comparison skeletal muscle fibre diameter and cross sectional area

Variable /Groups	**Hip fracture**	**Hip replacement**	**Healthy controls**	**p value**
Minimal ferret diameter (min ferret dm)				
Min ferret dm (µm) (all fibres)	49.62±8.3	47.52±6.7	51.79±7.6	0.41
Min ferret dm (µm) (MHC1 fibre)	55.09±7.2	52.11±8.9	59.00±9.8	0.16
Min ferret dm (µm) (MHC2 fibre)	40.98±10.4	40.47±7.7	47.03±8.1	0.13
Cross sectional area (CSA µm^2)				
CSA (µm^2) (all fibres)	3073±949	2794±778	3343±971	0.34
CSA (µm^2) (MHC1)	3678±915	3289±1076	4177±1276	0.16
CSA (µm^2) (MHC2)	2085±927	2039±820	2794±1017	0.08

MHC1: Myosin heavy chain 1/Type I muscle fibre, MHC2: Myosin heavy chain 2/Type II muscle fibre

Figure 5.5.6a: Muscle fibre diameter (minimal ferret dm) comparison between groups. Error bars are reported as SEM

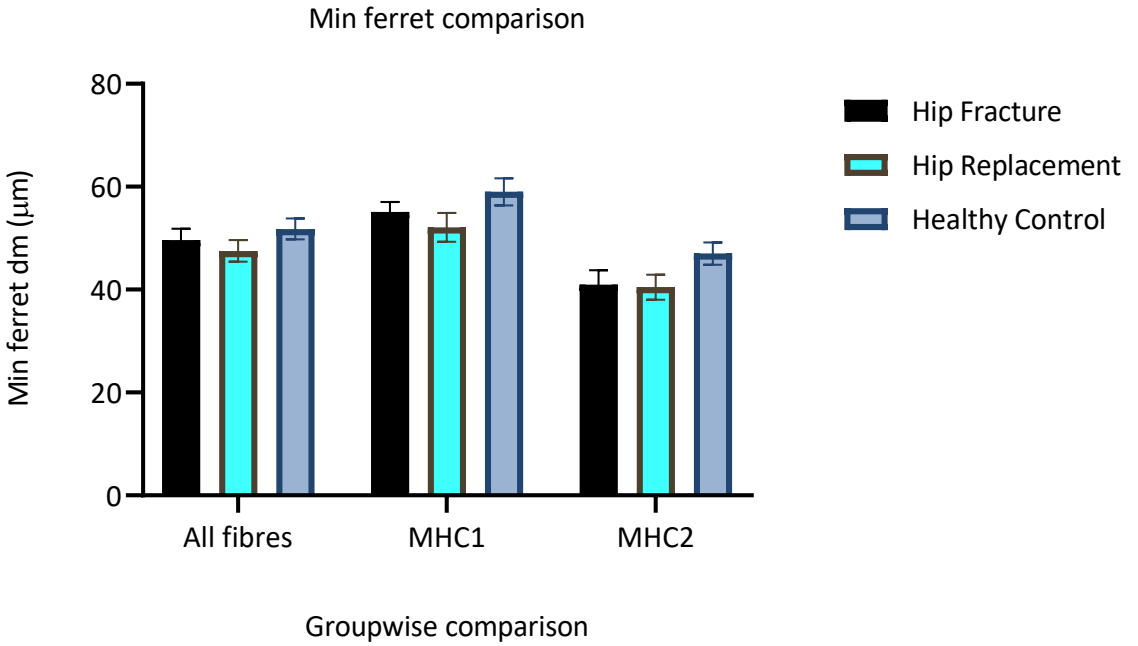
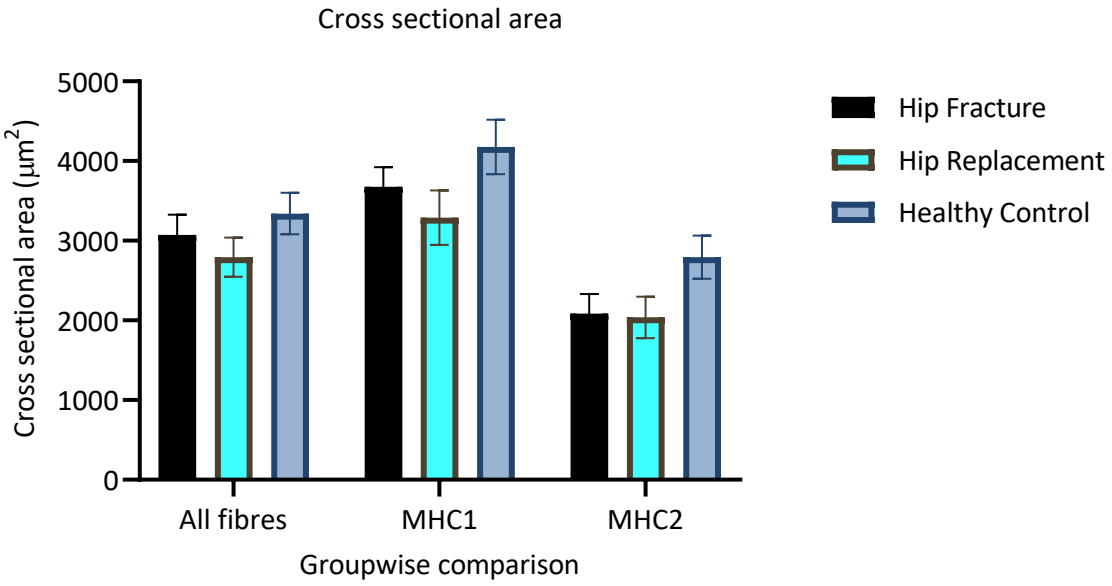


Figure 5.5.6b Muscle fibre cross sectional area comparison between groups. Error bars are reported as SEM



Muscle fibre type percentage between groups

Healthy controls had a smaller percentage of Type I muscle fibre and consequently a higher percentage of Type II muscle fibres. In contrast, women with hip fracture and hip replacement had a higher percentage of Type I and consequently a lower percentage of Type II muscle fibre ($p<0.01$) (Table 5.5.6b, Figure 5.5.6c).

Table 5.5.6b Percentage muscle fibre type comparison between groups

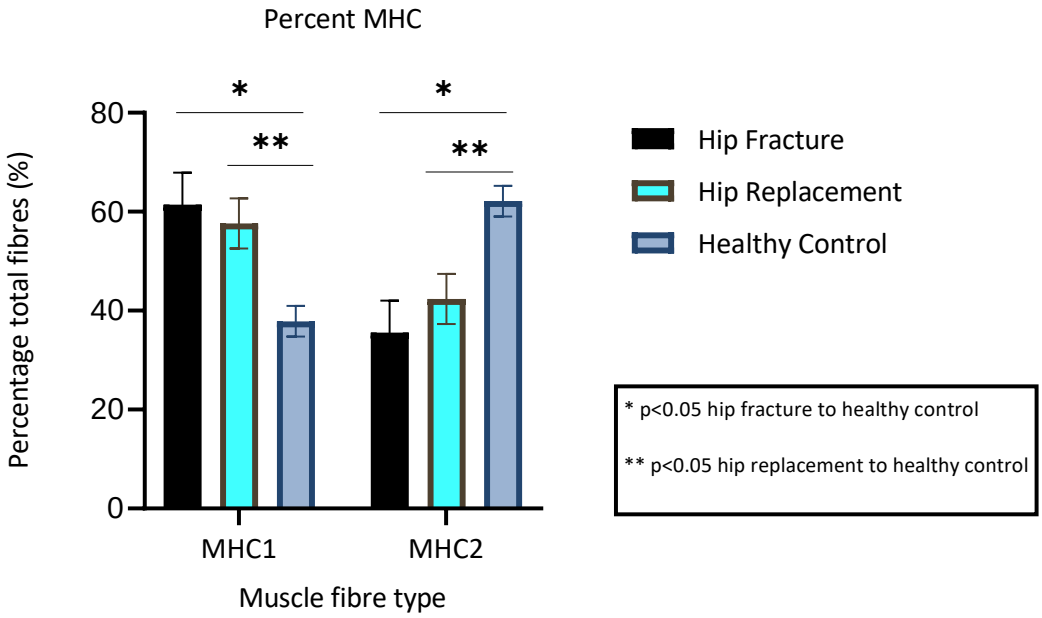
Variable /Groups	Hip fracture	Hip replacement	Healthy controls	p value
Per cent muscle fibre type (%)				
MHC 1 (%)	61.41±24.2 [a]	57.63±16.0 [b]	37.84±11.6 [a b]	<0.01
MCH 2 (%)	35.59±24.2 [a]	42.37±16.0 [b]	62.16±11.6 [a b]	<0.01

MHC1: Myosin heavy chain 1/Type 1 muscle fibre, MHC2: Myosin heavy chain 2/Type 2 muscle fibre

[a] p value < 0.05 comparison hip fracture to healthy controls

[b] p value < 0.05 comparison hip replacement to healthy controls

Figure 5.5.6c: Percent muscle fibre type comparison between groups



As the groups differed by age, ANCOVA analysis was performed adjusted for age as a confounder in the analysis of Type II muscle fibre percentage between groups. This is reported in Table 5.5.6c. Type II muscle fibre percentage in healthy controls remained significantly higher than women in the other groups with a calculated variance of 71.9% when adjusted for age.

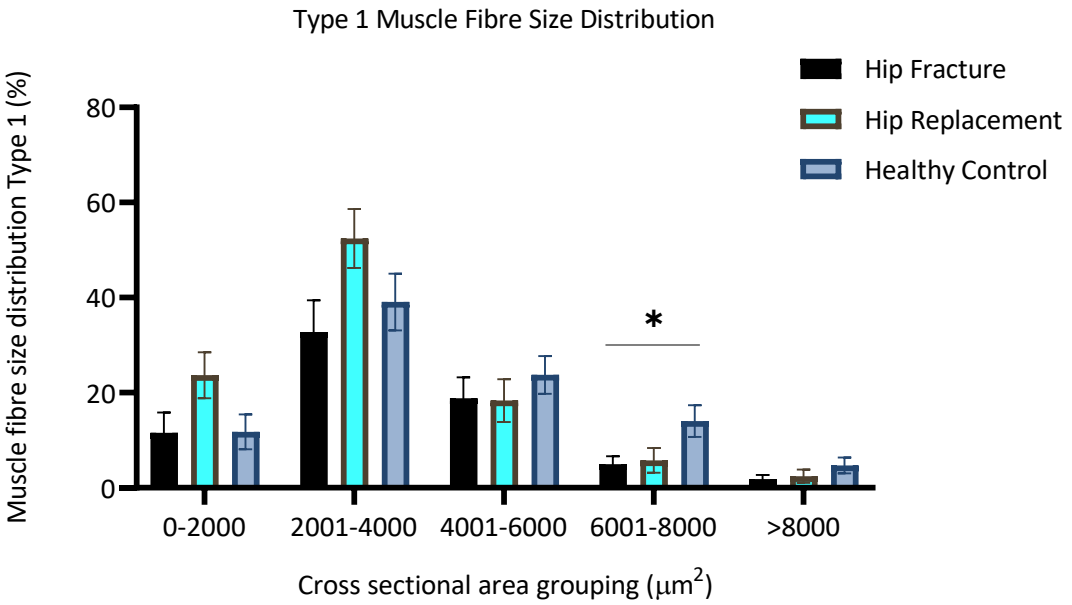
Table 5.5.6c: ANCOVA analysis for Type II muscle fibre percent between groups adjusted for age.

Variable	**p value**	**Partial ETA squared**
Age	0.02	0.958
Hip fracture status	0.02	0.719
Estimated marginal means		
Group	**Mean percentage Type II muscle fibre when adjusted for age**	
Hip fracture	38.59%	
Hip replacement	40.92%	
Healthy controls	61.09%	

Muscle fibre type distribution (frequencies) between groups

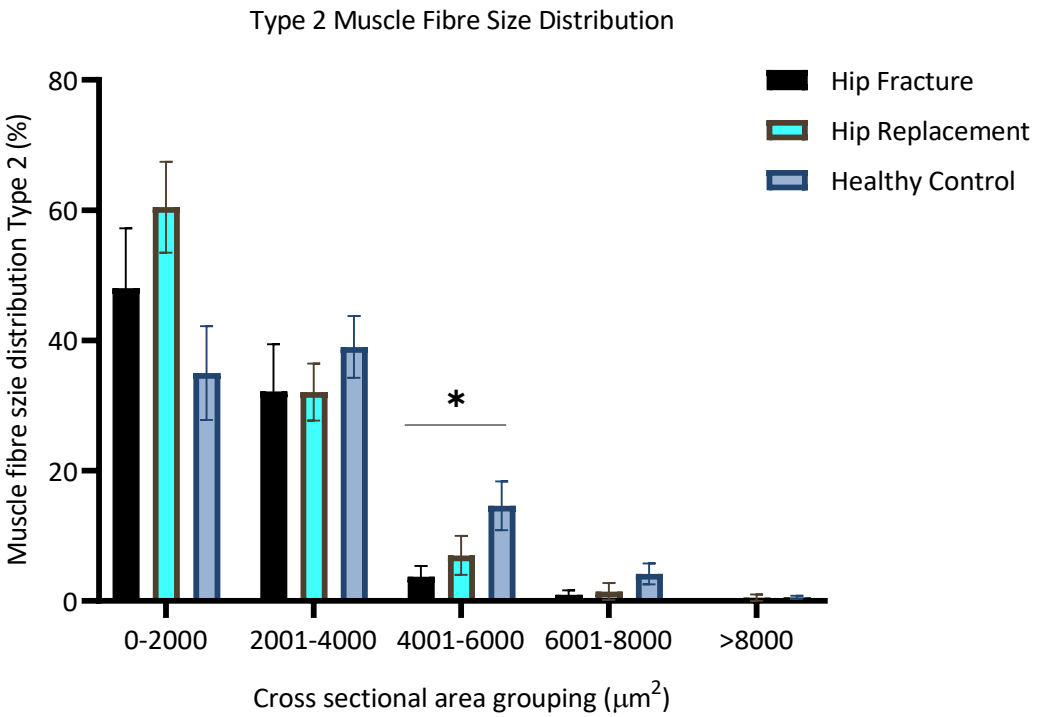
This is reported in Figures 5.5.6d and 5.5.6e. There was shift towards larger Type I and Type II muscle fibres in healthy controls compared to women with hip fracture and hip replacement. This difference was significant between the hip fracture group to healthy controls when comparing Type I muscle fibres between 6001-8000 μm^2 and Type II muscle fibres between 4001-6000 μm^2.

Figure 5.5.6d: Frequency of distribution of Type I skeletal muscle fibre size between groups



* p value <0.05 Hip fracture to healthy controls

Figure 5.5.6e: Frequency of distribution of Type II skeletal muscle fibre size between groups



* p value <0.05 Hip fracture to healthy controls

5.5.7 Relationship between muscle fibre parameters to measures of muscle mass and strength, sarcopenia status and hormonal markers (androgens, IGFBP3, IGF-1, HOMA Beta)

Relationship between muscle fibre parameters to measures of muscle mass and strength

Increased Type II muscle fibre measurements was associated with increased skeletal muscle mass and grip strength, whilst increased Type I muscle fibre was associated with lower muscle mass by CT and grip strength (Table 5.5.7a).

Table 5.5.7a Univariate regression analysis between muscle fibre parameters to measures of muscle mass and strength

Measures of skeletal muscle mass and strength				
Muscle fibre parameter	**Muscle mass or strength**	**Beta (95%CI)**	**r2**	**p value**
Min ferret MHC2	Muscle CT (cm^2)	0.751 (0.082, 1.420)	0.129	0.03
Percent MHC2		0.315 (0.000,0.629)	0.105	0.05
Percent MCH2	Grip strength (kg)	0.128 (0.011, 0.246)	0.119	0.03

Relationship between muscle fibre parameters to sarcopenia status

Binary logistic regression was performed assessing the relationship between muscle fibre percentage to sarcopenia status (Table 5.5.7b). Increased Type I muscle fibre was associated with increased odds of sarcopenia, conversely the odds of sarcopenia is lower with increasing percentage of Type II muscle fibre. For every increase in the percentage of Type I muscle fibre the odds of sarcopenia increases by a factor of 1.05.

Table 5.5.7b Binary logistic regression between percent muscle fibre to sarcopenia status

Binary logistic regression to sarcopenia status by EWGSOP1 criteria				
Category	**r^2**	**Beta (95% CI)**	**Odds ratio**	**p value**
Percent MCH2	0.184	-0.049 (0.91,0.99)	0.95	0.02

Relationship between muscle fibre parameters to hormonal markers

To determine if there was a relationship between hormonal levels involved in muscle regulation to muscle fibre count, cross sectional area, and measures of skeletal muscle mass and strength, univariate regression analysis was performed assessing the relationship between these different variables (Table 5.5.7c). Increased free testosterone levels were associated with lower Type I muscle fibre count, while HOMA beta was positively associated with Type II muscle fibre cross sectional area. There was a positive relationship between dihydrotestosterone, insulin and HOMA beta to measures of Type II muscle fibre percentage, and an inverse relationship to Type I muscle fibre percentage.

Table 5.5.7c Univariate regression analysis between hormonal levels to muscle fibre parameters

Hormonal markers				
Hormonal marker	**Muscle fibre parameter**	**Beta (95% CI)**	**r^2**	**p value**
Free testosterone	MHC1 count	-28.92 (-57.23, -0.61)	0.116	0.05
HOMA Beta	MHC2 cross sectional area	0.008 (0.000,0.016)	0.103	0.05
Insulin	Percent fibre MHC2	0.091 (0.013, 0.168)	0.136	0.02
HOMA Beta		0.406 (0.031, 0.782)	0.118	0.04
Dihydrotestosterone		0.001 (0.00,0.002)	0.309	0.05

MHC1: Type I muscle fibre, MHC2: Type II muscle fibre

5.6 Discussion

This study examined and compared the signalling proteins involved in skeletal muscle regulation in women with hip fracture to women awaiting total hip replacement and healthy controls. There were several observations which may provide insights into muscle regulation in this group of women. Initial hypothesized changes in signalling proteins and results from western blot analysis from this study are shown in Table 5.6a. As mean levels of signalling

proteins were reported as fold change, the trajectory of these levels are listed in comparison to the healthy control group.

Table 5.6a Comparison hypothesized versus study results in women with hip fracture and hip replacement in relation to healthy controls.

Signalling proteins	Hypothesized observations in women with hip fracture and hip replacement	Study findings in women with hip fracture and hip replacement compared to healthy controls
mTOR pathway		
mTOR/pmTOR	↓	↔
AKT/pAKT	↓	↔
RibS6/pRibS6	↓	↔
t 4EBP1	↑	↑
p4EBP1	↓	↓
Androgen, TGF-β pathway		
tSMAD 3/pSMAD 3	↑/↑	↑↑/↑
SMAD 7	↓	↑
Androgen receptor	↓	↔
Atrophy pathway		
MURF-1	↑	↑

t:total, p:phosphorylated

5.6.1 mTOR pathway

In contrast to the initial hypothesis there was no significant difference detected between groups in this pathway, except for higher levels of total 4EBP1 and lower phosphorylated to total 4EBP1 ratio in the hip fracture and hip replacement group. Upregulation of AKT/mTOR pathway in animal studies have been shown to be crucial in muscle growth and hypertrophy. Furthermore, it may also protect against muscle atrophy [428]. In the same study using denervated muscle fibres, tibialis anterior muscle which were injected with an activated form of AKT were found to have significantly larger muscle fibres compared to controls, supporting the protective role of AKT in preventing muscle atrophy.

This finding however was contrasted by a recent study [429] whereby inhibition of mTOR signalling in adult sedentary mice did not result in muscle atrophy. While the role of mTOR in muscle hypertrophy is well established, its function in muscle maintenance particularly the older muscle remains to be elucidated.

Human studies by Sandri in the older population have observed no significant difference in mTOR, AKT, 4EBP1 and Ribosomal S6 levels in muscles obtained from both young and old (active and sedentary) individuals [266]. Our study findings are in keeping with Sandri's study with the exception of 4EBP1 levels. The discrepancy in 4EBP1 levels between our study and Sandri's study may relate to the variation in study population. As their study participants were recruited from the community it is likely that they have a healthier and less sarcopenic population, compared to our women in the hip fracture and hip replacement group who were more sarcopenic.

In the current study, reduced phosphorylated to total 4EBP1 observed in the hip fracture and hip replacement groups supports the hypothesis that these are a group of sarcopenic women, as reflected by lower muscle area and grip strength (Chapter 3). It also suggest that the mTOR pathway is relatively underactive in sarcopenic individuals as the proportion of phosphorylated 4EBP1 is restricted allowing unphosphorylated 4EBP1 to inhibit eIF4e and protein synthesis. Although 4EBP1 is activated downstream in the mTOR signalling pathway the absence of a significant difference observed in signalling proteins upstream may suggest an alternative pathway in the activation of 4EBP1 independent of mTOR. Several kinases are reported to phosphorylate 4EBP1 independent of mTOR [255] including the extracellular signal regulated kinase (ERK) pathway. Interactions between ERK with IGF-1 and IGFBP3 have been reported before in oncological studies [430, 431], while overexpression of phosphorylated 4EBP1 have been associated with a poorer prognosis in malignancies in humans [432].

Specifically on the role of 4EBP1 and the muscle, resistance exercises have been shown to increase phosphorylation of 4EBP1 in the younger population [433] while no difference in 4EBP1 phosphorylation was observed in the older group [434]. There is currently no data pertaining to 4EBP1 levels in older women with hip fracture or hip replacement. Given these findings there are some possible explanations to findings from this study.

The lack of 4EBP1 phosphorylation in the hip fracture and hip replacement group may be explained by impaired mobility and the lack of resistance exercises, impaired nutrition (as reported by low levels of albumin in Chapter 3), or a resistance in the activation of the mTOR pathway upstream. In contrast in women in the healthy control group, the increase in phosphorylated form of 4EBP1 may relate to higher IGF-1 and IGFBP3 levels interacting with 4EBP1 independent of mTOR pathway. Given the positive association between 4EBP1 ratios to IGF-1 and IGFBP3 understanding this interaction in skeletal muscle regulation in future studies is pertinent in dissecting the different pathways in muscle maintenance in older women.

5.6.2 Androgen receptor, TGF-β pathway

In Chapters 3 and 4, several androgens (free testosterone, 11-ketotestosterone and dehydroepiandrosterone) were observed to have a positive relationship to muscle mass and strength. In this chapter, phosphorylated SMAD 3 and total SMAD 3 levels were significantly higher in women with hip fracture compared to healthy controls. Additionally, total SMAD 3 levels were also noted to be elevated in the hip replacement group.

The role of myostatin, a member of the TGF-β family is well implicated in muscle wasting. Myostatin null mice have hypertrophied muscles [267]. This discovery have led to trials using myostatin inhibitor as a potential therapeutic option for sarcopenia [22]. Although

improved lean body mass was associated with the use of this drug, increase in functional outcomes remains modest and requires further exploration.

Elevations in total SMAD 3 and phosphorylated SMAD 3 levels in the hip fracture and hip replacement group in this study are in keeping with previous studies on the upregulation of SMAD 3 in muscle atrophy [435, 436]. In humans with hip fracture, this finding have not been reported before and thus serves as new knowledge. Given the association between TGF-β and fibrosis [437], a comparison of fibrosis in muscle samples between groups was performed. Although there was no statistical difference detected between groups, images obtained from individuals with the highest degree of fibrosis from each group suggests that muscle fibrosis were highest in the two individuals with hip fracture and hip replacement. As the cause of skeletal muscle fibrosis is multifactorial [438], including increased levels of inflammation with the presence of tumour necrosis factor and interleukin 6, the unavailability of these results limits further comparison and may explain the lack of difference seen between groups.

An unexpected finding were elevated SMAD 7 levels in women with hip fracture and hip replacement, and the negative relationship between SMAD 7 to androgens. SMAD 7 is an inhibitory group of the MAD related protein and functions as a negative regulator of TGF-β signalling [439]. In studies using cell culture or male mice models, SMAD 7 is upregulated by testosterone [206] and increases skeletal muscle differentiation through the inhibition of myostatin [440]. However, the role of SMAD 7 and its interaction to the muscle in older women is not known.

There are several alternative explanations to findings from this study. As the role of SMAD 7 is to inhibit TGF-β through a negative feedback loop [273], upregulation of SMAD 7 levels may be a response to counteract the increase in phosphorylated SMAD 3 and total SMAD 3 levels. Alternatively increased SMAD 7 levels have also been reported with laminar shear

stress [439] and may suggest an underlying inflammatory process in these women. in view of the negative relationship between SMAD 7 and androgens it is possible that SMAD 7 may be upregulated in response to low androgen levels in these women.

Although SMAD 7 commonly inhibits TGF-β signalling, SMAD 7 also regulates Wnt and beta catenin signalling [273]. The Wnt signalling pathways are predominantly involved in tissue repair and have been studied in the regulation of carcinogenesis [441]. Evidence now shows that Wnt signalling also have roles in skeletal muscle growth and regeneration [442, 443]. Upregulation of the SMAD 7:beta catenin complex have been shown to regulate myogenic gene transcription[425] while activation of the Wnt signalling results in increased follistatin expression and myogenic differentiation [444].Given SMAD 7's role in myogenic differentiation, elevated SMAD 7 levels in the hip fracture and hip replacement group may also be a response to try and increase muscle production in these sarcopenic women. In view of the multiple interactions between SMAD 7 and the different pathways independent of TGF- β signalling, a better understanding of these interactions may provide further insights.

5.6.3 Atrophy, MURF-1 pathway

MURF-1 levels were significantly higher in women with hip fracture and hip replacement compared to healthy controls. In addition, the strong signal between MURF-1 to markers of skeletal muscle mass, strength, and sarcopenia status points towards increased MURF-1 activity as a predominant process of muscle loss in these women. MURF-1, together with atrogin-1 are muscle specific E3 ubiquitin ligases which function to polyubiquitinate labelled proteins for subsequent proteolysis. Upregulation of MURF-1 and atrogin-1 levels are reported in animal and human models of muscle atrophy [259]. Conversely in another study utilizing denervation and disuse in animal models downregulation of MURF-1 levels have also been reported. This suggests underlying mechanisms to sarcopenia are different from that observed with denervation and disuse atrophy [445]. MURF-1 is also driven by FOXO

which is stimulated by the SMAD pathway and inhibited by AKT. Given the lack of activity in protein signalling in AKT, and the increase in total SMAD 3 levels, upregulation of MURF-1 in the sarcopenic group may be via the FOXO pathway.

Not many studies have been performed in hip fracture patients assessing MURF-1 expression. In a study by Bamman [446], they compared the effect of muscle inflammation on recovery in patients with hip fracture, hip replacement and healthy controls. MURF-1 expression did not differ between groups. In their cohort of hip replacement patients who were analysed separately, MURF-1 levels were noted to be three-fold higher in those with increased markers of inflammation indicating that increased MURF-1 activity was associated with increased inflammation.

Increase age is associated with increased inflammatory process and may explain the elevated MURF-1 levels seen in our cohort. However, it is important to note that patients in Bamman's study were younger (mean age 44-50 years) and consisted of men and women. As increased MURF-1 levels have been reported in older women before [305, 447] expression of MURF-1 levels may be gender specific and thus requires further exploration.

5.6.4 Muscle fibre type distribution

Women in the hip fracture and hip replacement groups appeared to have a higher percentage of Type I muscle fibre while healthy controls have a higher proportion of Type II muscle fibre, independent of age. There was also a shift towards smaller Type I and II muscle fibres in women with hip fracture and hip replacement while healthy controls had larger muscle fibres. This finding suggests women with hip fracture and hip replacement not only have a greater propensity for smaller Type I and Type II muscle fibres but also a shift towards a higher proportion of Type I muscle fibres.

While aging is associated with Type II muscle fibre atrophy [161], this observation was maintained independent of age in this cohort suggesting there are other factors apart from increase age to explain the loss and atrophy in Type II muscle fibre. Given the positive association between insulin, HOMA beta and dihydrotestosterone to Type II muscle fibre proportions it is likely these hormones play a key role in the shift towards Type II muscle fibre. Atrophy in Type II muscle fibre in women with hip fracture from this cohort are in keeping with findings from another study in women with hip fracture [162].

Strengths and limitations:

The strength of this study is that of the investigation of a series of intracellular signalling pathways involved in muscle regulation providing new knowledge on the activity of these pathways in older women. The main findings from this study are MURF-1 as a predominant role in muscle atrophy in these women, and the high levels of SMAD 7 likely as a compensatory response to high SMAD 3 levels in older women with hip fracture compared to women awaiting total hip replacement and healthy controls. Higher unphosphorylated 4EBP1 also contributes to reduced muscle protein synthesis in the sarcopenic groups. These findings provide new information which can be used to further examine the dynamics of these pathways in muscle regulation in older women.

There were however limitations to the study findings. Information on nutritional status and physical activity, factors which are known to affect skeletal muscle mass and strength was not obtained from the participants. As mTOR is known to downregulate in response to impaired nutrition this may explain the lack of activity seen in mTOR signalling in this study. Additionally, as MURF-1 levels may be related to increased inflammation, the lack of inflammatory markers further limits comparison between groups.

5.7 Conclusion

- Western blot analysis shows no change in mTOR signalling while a reduction in phosphorylated to total 4EBP1 was observed in women with hip fracture and hip replacement. A pathway independent of mTOR signalling may be the underlying mechanism to this observation and lies in the interaction between serum IGF-1, IGFBP3 and 4EBP1

- Increased TGF-β signalling particularly total and phosphorylated SMAD 3 levels appears to be higher in women with hip fracture and hip replacement

- SMAD 7 levels were unexpectedly higher in women with hip fracture and hip replacement and indicate a compensatory mechanism to increased signalling proteins in the TGF-β pathway in older women

- MURF-1 levels were consistently associated with lower skeletal muscle mass by various measures, strength, and sarcopenia status, and is likely to be the key driver of muscle atrophy in this group

- Atrophy in both Type I and II muscle fibres were observed in the hip fracture and hip replacement group. Type II muscle fibre atrophy are consistent with previous reports in hip fracture patients. Insulin, HOMA Beta and dihydrotestosterone were positively associated with increased Type II muscle fibres.

- Women in the hip replacement group were recruited as they were considered to be less sarcopenic than the hip fracture group. However increasing evidence from this study suggests women awaiting hip replacement were as sarcopenic as women with hip fracture and share some similarities in terms of reduction in hormonal levels, increase signalling proteins in muscle atrophy and reduced Type II muscle fibre. This may indicate a need to consider early intervention although this needs to be explored in a separate study.

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5.8 Appendix

Step by step methods for protein extraction and western blot

Protein extraction: Muscles were weighed and sections of 25-30 milligrams of muscle samples were processed in the chipping chamber. For protein extraction, total buffer solution required was calculated at a ratio of 15 microlitre (µcl) buffer solution for every 1 miligram (mg) of muscle sample. The final buffer solution made consists of the following components:

- 10 X radioimmunoprecipitation assay (RIPA) buffer (Milipore, Temecula, CA USA)
- 1000 X protease inhibitor cocktail (Sigma Aldrich, Castle Hill Australia)
- 100 X Halt phosphatase inhibitor (Thermo Fisher Scientific, Rockford USA)
- Mili Q Ultrapure water

The buffer solution and was added to the muscle samples and vortexed before it was homogenized using a pestle. Samples were left in a spinner overnight in a 4° C fridge before processing the following day.

Tissue samples were then centrifuged for 15 mins at 13000 revolutions per minute (rpm) at 4 °C. The supernatant was collected into new labelled Eppendorf tubes and cell pellet discarded. 5 µcl of supernatant were diluted in 495 mcl of Mili Q water to a ratio of 1:100 and frozen in a -80 °C freezer.

Protein Standard Curve Preparation: For a standard curve preparation, a bicinchoninic acid (BCA) working reagent (Thermo Fisher Scientific) was prepared using Reagent A and Reagent B depending on the number of wells required based on the following calculation:

Volume of Reagent A = 150 µcl X number of wells

Volume of Reagent B = (150 µcl X number of wells)/50

A standard stock of Bovine Serum Albumin (BSA) was also prepared with serial dilutions performed to obtain the following concentrations:

1. 120 mcl BSA stock +1380 µcl Mili Q water: 160 ng/µcl
2. 750 mcl 160ng/µcl +750 µcl Mili Q water: 80 ng/µcl
3. 750 mcl 80ng/µcl +750 µcl Mili Q water: 40 ng/µcl
4. 750 mcl 40ng/mcl +750 µcl Mili Q water: 20 ng/µcl
5. 750 mcl 20ng/mcl +750 µcl Mili Q water:10 ng/µcl
6. 750 mcl 10ng/mcl +750 µcl Mili Q water: 5 ng/µcl
7. 750 mcl: 0 ng/µcl

In a 96 well plate, 150 µcl of BCA working reagent (Containing both Reagent A and B) was pipetted into all wells.

150 µcl of the BSA stock (diluted to different concentrations listed above), in addition to diluted supernatant from muscle samples were pipetted in triplicates into a 96 well plate which was prefilled with 150µcl of BCA working reagent. Figure 5.8a shows a map of how the well plate was filled.

Figure 5.8a: Map of how the 96 well plate was filled.

0ng/µcl BSA	0ng/µcl BSA	0ng/µcl BSA	Sample 2	Sample 2	Sample 2	Sample 10	Sample 10	Sample 10	Sample 18	Sample 18	Sample 18
5ng/µcl BSA	5ng/µcl BSA	5ng/µcl BSA	Sample 3	Sample 3	Sample 3	Sample 11	Sample 11	Sample 11	Sample 19	Sample 19	Sample 19
10ng/µcl BSA	10ng/µcl BSA	10ng/µcl BSA	Sample 4	Sample 4	Sample 4	Sample 12	Sample 12	Sample 12	Sample 20	Sample 20	Sample 20
20ng/µcl BSA	20ng/µcl BSA	20ng/µcl BSA	Sample 5	Sample 5	Sample 5	Sample 13	Sample 13	Sample 13	Sample 21	Sample 21	Sample 21
40ng/µcl BSA	40ng/µcl BSA	40ng/µcl BSA	Sample 6	Sample 6	Sample 6	Sample 14	Sample 14	Sample 14	Sample 22	Sample 22	Sample 22
80ng/µcl BSA	80ng/µcl BSA	80ng/µcl BSA	Sample 7	Sample 7	Sample 7	Sample 15	Sample 15	Sample 15	Sample 23	Sample 23	Sample 23
160ng/µcl BSA	160ng/µcl BSA	160ng/µcl BSA	Sample 8	Sample 8	Sample 8	Sample 16	Sample 16	Sample 16	Sample 24	Sample 24	Sample 24
Sample 1	Sample 1	Sample 1	Sample 9	Sample 9	Sample 9	Sample 17	Sample 17	Sample 17	Sample 25	Sample 25	Sample 25

The filled 96 well plate was incubated at 37 °C for 35 minutes and was further scanned for absorbance at 562 nanometer (nm). Information obtained were used to calculate the amount of protein concentration required from the aliquots to load into the western blot wells. Aliquots of 30 microgram (µcg) of protein from each diluted supernatant sample was made in preparation for western blot procedure. The resulting protein aliquots were made to yield a concentration of 4 µcg/µcl. To obtain at least 30 µcg of protein, 7.5 µcl of the 4µcg/µcl of protein aliquots were prepared.

Western Blot:

Materials:

Bio Rad Precision Plus All Blue Standards Biorad USA (Ladder)

Invitrogen NuPage sample buffer (Lamelli Dye)

Invitrogen NuPage reducing agent

Bio Rad 10X Tris/Glycine/SDS (Running buffer)

Bio Rad 10XTris/Glycine (Transfer buffer)

Bio Rad Criterion TGX Stain free Precast Gel 4-15% (Pre-cast Gel)

Immobilon P-PVDF-FL (membrane)

Filter paper

Protein separation:

i. A solution of 4X Lamelli Dye and 10X reducing agent was made and added to protein aliquots. The mixture was denatured at 90° Celsius for 5 minutes and then kept on ice.

ii. A running buffer solution was made with 100ml 10 X running buffer and 900 ml Mili Q water

iii. A transfer buffer solution was made with 100 ml 10 X transfer buffer combined with 200ml Methanol and 700 ml Mili Q water and refrigerated at -20 ° Celsius to cool.

iv. The pre-cast gel contained in a plastic mould is rinsed in deionized water and placed into a tank, filled with running buffer solution made earlier. The comb in the pre-cast gel is removed and flushed with running buffer.

v. 4 µcl of blue standard ladder is loaded into the first and last well, followed by the protein aliquots which have already been mixed with Lamelli dye and reducing agent prior.

vi. The electrode of the tank is connected to an electrophoresis machine and run at 200 volts for 40 minutes.

vii. The pre-cast gel in the plastic mould is removed from the tank, and gently transferred onto the Bio Rad Gel Doc machine (Image Lab software) to be read. The quantification of total protein loaded onto the gel through electrophoresis is obtained through this procedure.

Transfer of protein onto membrane:

I. The gel is placed into cold transfer buffer prepared earlier.

II. An Immobilon P-PVDF-FL membrane is cut in dimensions of 13 X 8.5cm. 4 filter paper in the same dimensions are also prepared, and soaked in transfer buffer.

III. Activation of the membrane is performed with embedding it in methanol for 30 seconds, then Mili Q water for 10 seconds and soaked in cold transfer buffer solution.

IV. The assembly chamber is filled with cold transfer buffer and a sandwich in the following layers are prepared: sponge, 2 filter paper, PVDF-FL membrane, gel from electrophoresis performed earlier, 2 filter paper, sponge.

V. The sandwich is placed into the transfer container. An ice block is placed next to the sandwich in the transfer container. The transfer container is filled with transfer buffer until the sandwich is submerged in the solution. The transfer container is placed in an esky filled with ice and electrodes connected to the transfer machine and run at 100 volts for 30 minutes.

VI. Following transfer, the membrane is removed from the sandwich and sections of the membrane is cut with a scalpel.

Blocking antibody and primary antibodies:

Materials:

Devondale Skim milk (Blocking antibody)

Bovine Serum Albumin Bovogen Biologicals Keilor East, Victoria (Primary Blocking antigen)

10 X TBST (Tris buffered saline Tween):

- 50 mM Tris base 6.05g
- 750 nM NaCl 43.5 g
- 0.5% Tween 20 2.5 ml

10 X Blocking Buffer (1X TBST with 5% skim milk powder):

- 10 x TBST 10ml
- Skim milk powder 5g
- 100ml miliQ water

Washing Buffer (1XTBST):

- 100 ml TBST
- 900 ml Mili Q water

I. The blocking antibody was prepared with either 4% skim milk or bovine serum albumin as recommended by the manufacturers

II. The membrane sections cut earlier are embedded in blocking antibody for 1 hour at room temperature on a rocker

III. The primary antibody is prepared in 5% skim milk or bovine serum albumin and TBST at a concentration of 1: 1000.

IV. After 1 hour, the blocking antibody is discarded, and membrane embedded in the primary antibody and incubated at 4 ° Celsius on a rocker overnight.

Washing and secondary antibody

I. The following day, the membrane is washed in TBST (washing buffer) 3 X 10 minutes

II. Secondary antibodies are made with either 5% skim milk or BSA with TBST at 1:10 000.

III. The membrane is embedded with the secondary antibodies and incubated at 1 hour at room temperature in the dark.

IV. The membrane is transferred onto the Licor Image Scanner to be read and protein quantified

Chapter 6: Summary, integrative discussion and future directions

Sarcopenia is a dynamic and complex condition encompassing different interactive pathways. It is influenced by the presence of different hormones and external factors (nutrition, physical activity) which makes sarcopenia management all the more challenging. Despite the availability of current guidelines for sarcopenia diagnosis, access to these tools may be limited in a clinical setting. Moreover successful pharmacological treatment options for sarcopenia remains to be discovered.

The general aims of the thesis were to

1) Explore the use of simple anthropometry measures as a screening tool for sarcopenia screening in a clinical setting where access to diagnostic imaging may be limited

2) Assess the relationship between lean mass to bone strength, and fat mass to bone strength in a group of Australian women with heterogenous medical conditions

3) Characterise the hormonal profile and identify potential biomarkers for low skeletal muscle mass in a group of sarcopenic Australian women with hip fracture compared to those awaiting total hip replacement and healthy controls

4) Compare an expanded sex steroid profile including a group of adrenal androgens, and examine the relationship between adrenal androgens to muscle regeneration as evidenced by the presence of centralised skeletal muscle nuclei

5) Characterise and compare the different signalling pathways in muscle regulation and its relationship to several hormonal biomarkers of sarcopenia

In Chapter 2, calf circumference were shown to be a good predictor of skeletal muscle mass by DEXA in a select population of Australian women. A calf circumference of less than 35.6 cm provide a sensitivity of 88.6% and specificity of 54.5% when detecting low skeletal muscle mass by EWGSOP 2 criteria. In view of the lack of Australian specific cut points, these findings provide an opportunity to further examine and compare its utility as a simple bedside screening tool for low skeletal muscle mass in populations where access to specific skeletal muscle imaging may be limited.

On the same cohort, older women were observed to be shorter when leg length appear stable across age groups. Comparisons of the skeletal muscle index using both height and leg length as the denominator shows leg length adjusted SMI (LL-SMI) were as effective as height adjusted SMI (H-SMI) in detecting SMI differences between younger and older women. Given the parallel decline in height with age, the use of H-SMI may obscure and underestimate sarcopenia, particularly in the older population. In this setting LL-SMI may be more useful and therefore warrants further exploration.

Fat and lean mass were both significant in its association with bone mineral density in this heterogeneous group of women who presents with several medical conditions. When fat mass was adjusted for height, lean mass was a stronger predictor of bone mineral density. To contrast this with studies in healthy individuals where fat mass may be detrimental to bone [297, 300], findings from the current study highlight the importance of preserving both fat and lean mass especially in individuals with chronic medical conditions who are at risk of losing both fat and lean mass due to increased metabolic demands. In those with a higher proportion of fat relative to lean i.e. FMI > 20kg/m^2 preserving lean mass appear to be the predominant factor in determining bone strength.

The strength to this study were findings that were derived from analysis in a select cohort of women in Victoria Australia, providing data relevant to the local population. While the

limitations to this study were the analysis was performed on a select cohort of women, the lack of ethnicity, functional assessments and grip strength, it has contributed to the field by providing a comparative measure of calf circumference. It also highlights the need to explore leg length as a denominator in the SMI. Although lean and fat mass are important for bone strength, the degree to which fat mass may be detrimental to the bone may be dependent on its distribution as there are reports pointing towards the protective role of peripheral fat [345] while visceral fat is detrimental [323] to bone strength. Moving forwards, the key themes arising from this study for future analysis include:

- Large scale population studies examining the utility of several calf circumference cut points in detecting low skeletal muscle mass and its association with functional and clinical outcomes
- Exploring the utility of leg length as a denominator in the skeletal muscle index and its association with muscle function and clinical outcomes
- The association between lean mass, visceral and peripheral fat distribution and its effect on the bone comparing those with chronic wasting illness and healthy controls from the community

In Chapters 3 to 5, analysis was performed on a total of forty nine women consisting of women with hip fracture to women awaiting hip replacement and healthy controls (Cohort 2). The broad aims of these studies combined were to compare a group where there is a high proportion of sarcopenic individuals to a group where there is a lower proportion of sarcopenic individuals from several aspects: hormonal profile, histology analysis and investigation of the muscle regulatory pathways.

Using CT as a gold standard tool for low skeletal muscle mass combined with low grip strength, the hip fracture group had a higher proportion of people with sarcopenia and had lower measures of skeletal muscle mass when compared to women with hip replacement and healthy controls. In the exploration of hormonal profile in these women, low levels of

serum IGF-1, IGFBP3, testosterone and impaired beta cell function were detected in the hip fracture group. While IGF-1 and testosterone levels are known positive regulators of skeletal muscle, the association between IGFBP-3, impaired beta cell function and skeletal muscle in women with hip fracture is a novel finding. This supports their role as potential biomarkers of sarcopenia where access to CT, DEXA or grip strength testing may be limited.

In Chapter 4, when exploring the sex steroid profile, levels of the 11-oxygenated steroids were lower in women with hip fracture. 11-ketotestosterone was associated with increased muscle function as measured by grip strength. Low androgen levels were associated with the presence of high central nuclei count indicating increase muscle regeneration as a response to low androgen levels, a contrasting finding to studies in men where increased androgen levels were associated with increased muscle regeneration.

An unexpected finding was the elevated oestrone levels in the hip fracture group despite a higher proportion of older women suggesting increased peripheral aromatisation with acute illness, consistent with observations in ICU patients. Furthermore when comparing women with hip replacement to healthy controls, significantly higher oestrone levels were detected in the less sarcopenic, healthy control group. This raises the question of two possible separate mechanisms observed in these women. Elevated oestrone levels in a stressed state (hip fracture) and long standing higher oestrone levels in the least sarcopenic group in a non-stressed state (healthy controls) indicating oestrone may have positive role to play in skeletal muscle growth. To support this hypothesis, there are evidence to suggest the presence of oestrogen receptors in human skeletal muscle [396], and HRT users were observed to have preserved muscle mass and increase muscle strength compared to their non HRT counter parts [448, 449] .

In Chapter 5, several signalling proteins were the highlight of the study when examination of the muscle regulatory pathways were performed. The marked elevation in SMAD 7 in

response to SMAD 3 levels, the significant elevation in MURF-1 levels in the hip fracture group and its strong signal to low muscle mass and strength, and the association between IGFBP-3 to 4EBP3 ratios. This has not been reported in women with hip fracture before and points towards the likely mechanisms in muscle regulation in this group. Contrary to study findings in male models where SMAD 7 were positively associated with androgens [206], the study shows that in women elevated SMAD7 levels were associated with low androgen levels. This points towards SMAD7 as a compensatory response to elevated SMAD 3 levels and to low androgen levels in older women. Elevated MURF-1 levels in the hip fracture and hip replacement group provided a strong signal to measures of skeletal muscle mass and strength supporting its role as a major pathway in muscle atrophy in these women.

There were some limitations to consider. While the study was adequately powered to detect a significant difference in muscle area by CT, the small sample size remains a limiting factor in the generalizability of study findings. However from the perspective of laboratory analysis for histology and signalling proteins using muscle biopsy samples, these numbers were similar to other studies examining muscle biopsy samples [290, 396, 450] and thus remains relevant in providing a snapshot to the underlying mechanisms to muscle regulation in these women. In terms of hormonal profile, the lack of serial hormonal testing does limit the ability to assess the progression and trajectory of these hormonal levels in the recovery phase particularly in those who were acutely unwell (hip fracture group). This is an important lesson learnt in the course of performing this research project.

While women awaiting total hip replacement were initially recruited for the study for convenience sampling of muscle biopsy, it has emerged that these group of women share some similarities and were as sarcopenic as women in the hip fracture group (low muscle area and increased MURF-1 level). Although the primary aim in our study was not to examine sarcopenia in these women, important clinical implications and questions arising from this thesis are:

- The proportion of sarcopenia in this group of women pre and post-surgery and its implications in terms of recovery and outcomes
- What are the key regulatory pathways to muscle homeostasis in these women?
- Does early intervention in terms of pre-rehab [451, 452] for women in this group have any benefits or effects on the intracellular signalling pathways, recovery and improved function post operatively?

Combining findings from chapters 3-5, potential areas for future research are:

- A comparison of sex steroid profile including oestrone levels in a larger group of women with hip fracture with serial blood sampling to determine if changes in hormonal levels are associated with acute illness

- Following a group of perimenopausal women through the menopausal transition and examining the changes in muscle mass and function including muscle growth and atrophy pathway activation

- Exploring the role of aromatase inhibition on muscle mass and function

- The interrogation of cross signalling between SMAD7/SMAD 3/Androgens and its relationship to the IGFBP3/mTOR pathway in older women, expanding on our current recruited control women

- Exploring the role of the TGF beta family members and interaction with androgens in frail older women

- Therapeutic trials of precursor steroids such as DHEA and androstenedione, SARMS, assessing its efficacy in combination with nutrition and exercise in preventing sarcopenia and physical frailty

To conclude the thesis findings and contributions to the field:

1. Calf circumference is a good predictor of low muscle mass in a group of Australian women above age 40

2. Height is lower in older women compared the younger age group. This may obscure and underestimate skeletal muscle mass, particularly in the older population

3. Leg length appears unchanged across age and should be explored as a denominator in the skeletal muscle mass index

4. Women with hip fracture have high prevalence of sarcopenia, with low IGF-1, IGFBP3, serum testosterone and beta cell function

5. Of the 11-oxygenated androgens explored, 11-ketotestosterone is associated with grip strength in this group of women

6. Low androgen levels was associated with the presence of high centralised nuclei as a marker of skeletal muscle regeneration and reflects a compensatory response to low androgen levels in women

7. High SMAD 7 levels observed in the sarcopenic group is a compensatory response to elevated SMAD 3 and low androgen levels

8. Increased MURF-1 levels suggests this is the predominant pathway of muscle atrophy in older women

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