Development and application of minimally invasive stable isotope tracers' measures of muscle mass and muscle protein synthesis/breakdown: insights into ageing skeletal muscle

Jessica Cegielski

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

January 2020

Acknowledgements

I would like to thank my supervisors Professor Phil Atherton and Professor Kenneth Smith for their continual support, patience, supervision and guidance throughout my research. I would like to thank them for the numerous opportunities they have provided me to continually learn and expand my knowledge.

Thank you to my supervisor Professor John Gladman for his supervision and guidance, providing me a clinical outlook.

I would like to thank Dr Dan Wilkinson for his assistance and guidance with development of mass spectrometry methods. Thank you for all your perseverance and patience. Thank you to Dr Beth Phillips your support throughout and to Dr Matt Brook for your assistance in the lab.

I would like to thank Amanda Gates and all clinicians who helped out on the study days. Thank you to all the volunteers who took part and for making this research possible.

To Dr Colleen Deane, Dr Joe Bass, Dr Jonny Quinlan and Debbie Rankin for all of your support throughout. Could not have gone through this without you.

Thank you to the Abbeyfield Foundation for funding my PhD studentship.

Author Contributions

The author of this thesis has run the main study, prepared all samples and analysed the data presented in Chapter 4 and Chapter 5. The two studies described in Section 4.3.9. were run by Tessa Morris-Paterson (King's College London) and Nima Gharadaghi. The 3MH analyses in these studies were performed by J Cegielski. In Chapter 3, the DXA data was collated by a research colleague, Ruth Willott. Functional data from previous studies was collated by the author of this thesis. The analysis of data and statistics was performed by the author. Chapter 6 was a joint effort where Matthew Brook, Jonathan Quinlan, Daniel Wilkinson and Jessica Cegielski were involved in data collection and analysis. Daniel Wilkinson, Bethan Phillips, Philip Atherton and Kenneth Smith designed the study. J Cegielski was the first author for the manuscript.

Publications

Following list is my contribution during the PhD.

- **Cegielski J**, Brook MS, Quinlan JI, Wilkinson DJ, Smith K, Atherton PJ, Phillips BE. A *4-week*, lifestyle-integrated, *home-based* exercise training programme elicits improvements in physical function and lean mass in older men and women: a pilot study [version 2; referees: 2 approved]. *F1000Research* 2017, **6**:1235

- Wilkinson, D.J., Bukhari, S.S.I., Phillips, B.E., Limb, M.C., **Cegielski, J.,** Brook, M.S., Rankin, D., Mitchell, W.K., Kobayashi, H., Williams, J.P., Lund, J., Greenhaff, P.L., Smith, K., Atherton, P.J., 2017. Effects of leucine-enriched essential amino acid and whey protein bolus dosing upon skeletal muscle protein synthesis at rest and after exercise in older women. *Clinical Nutrition* 1–12. doi:10.1016/j.clnu.2017.09.008

- Wilkinson D.J., Hossain T., Limb M.C., Phillips B.E., Lund J., Williams J.P., Brook M.S., **Cegielski J.**, Philp A., Ashcroft S., Rathmacher J.A., Szewczyk N.J., Smith K. and Atherton P.J. Impact of the calcium form of

β-hydroxy-β-methylbutyrate upon human skeletal muscle protein metabolism. *Clinical Nutrition.* 2017 p1-8.

- Samuel G Impey; Kelly M Hammond; Robert Naughton; Carl Langan-Evans; Sam O Shepherd; Adam P Sharples; **Jessica Cegielski**; Kenneth Smith; Stewart Jeromson; D Lee Hamilton; Graeme L Close; James P Morton. Whey Protein Augments Leucinemia and Post-Exercise p70S6K1 Activity Compared to a Hydrolysed Collagen Blend When in Recovery From Training With Low Carbohydrate Availability. *International journal of sport nutrition and exercise metabolism.* 2018 DOI: 10.1123/ijsnem.2018-0054

- Sargeant JA, Bawden S, Simpson EJ, Aithal GP, MacDonald IA, Turner MC, **Cegielski J**, Smith K, Dorling JL, Gowland P, Nimmo MA, King JA. Effects of sprint interval training on ectopic lipids and tissue-specific insulin sensitivity in men with non-alcoholic fatty liver disease. *European Journal of Applied Physiology.* 2018 DOI: 10.1007/s00421-018-3818-y

- Olouyomi Gnimassou, Rodrigo Fernandez-Verdejo, Matthew Brook, Damien Naslain, Estelle Balan, Mariwan Sayda, **Jessica Cegielski**, Henri Nielens, Anabelle Decottignies, Jean-Baptiste Demoulin, Kenneth Smith, Philip J. Atherton, Marc Francaux and Louise Deldicque.. Environmental hypoxia favors myoblast differentiation and fast phenotype but blunts activation of protein synthesis after resistance exercise in human skeletal muscle. *The FASEB Journal. doi: 10.1096/fj.201800049RR*

- Nima Gharahdaghi, Supreeth Rudrappa, Matthew S. Brook, Iskandar Idris, Hannah Crossland, Clair Hamrock, Hariz Aziz, Fawzi Kadi, Paul L. Greenhaff, Dumitru Constantin-Teodosiu, Jessica Cegielski, Bethan E. Phillips, Daniel J. Wilkinson, Nathaniel J. Szewczyk, Kenneth Smith and Philip J. Atherton. Testosterone therapy induces molecular programming augmenting physiological adaptations to resistance exercise in older men. *Journal of Cachexia, Sarcopenia and Muscle.* 2019. DOI:10.1002/jcsm.12742

Book Chapter

- Philip J. Atherton, **Jessica Cegielski**, and Daniel J. Wilkinson (2019)., '20. Genetic contributions to skeletal muscle size' in J. Timothy Lightfoot, Monica J. Hubal, Stephen M. Roth. (ed.) *Routledge Handbook of Sport and Exercise Systems Genetics*. Routeledge

Presentations

Poster:

- Cegielski J, Wilkinson DJ, Brook MS, Boereboom C, Phillips BE, Gladman JFR, Smith K and Atherton PJ, 'Combined Oral Stable Isotope Assessment of Muscle' (COSIAM) approach to simultaneously quantify muscle mass and protein turnover: implications for ageing.;Physiology 2019, Aberdeen UK
- A 4-week "lifestyle-integrated" home-based resistance exercise training programme elicits improvements in physical function and increases lean mass; Physiology 2016, 29th-31st July 2016, Dublin, Ireland
- Can wee test for muscle-wasting?; Research Showcase 2016, University of Nottingham, UK

Oral:

- Development and application of minimally invasive stable isotope techniques to determine muscle protein synthesis, muscle protein breakdown and muscle mass simultaneously: relevance to ageing; Rank Prize Funds Mini-Symposium on Nutrition: Implications to ageing processes and people, 3rd-6th October 2016, Grasmere, UK.
- School of Medicine Oral Sue Watson PGR Presentation Event; Development of minimally invasive stable isotope techniques to determine muscle protein synthesis, muscle protein breakdown and muscle mass simultaneously: relevance to ageing

Awards

- 1st Prize at University of Nottingham Sue Watson PGR Presentation event
- Awarded a £3000 grant to organise the Young Life Scientists' Symposium 2017- Frontiers in Musculoskeletal Health, Ageing and Disease, Derby, UK, 2017

Table of Contents

1. INTRODUCTION 1

1.1. THE IMPORTANCE OF SKELETAL MUSCLE IN HEALTH, AGEING AND DISEASE 1

1.2. SKELETAL MUSCLE STRUCTURE 2

1.2.1. Structure overview 2

1.2.2. Muscle Fibres 4

1.3. MUSCLE PROTEIN TURNOVER 5

1.3.1. Muscle Protein Synthesis 5

1.3.2. Muscle Protein Breakdown 6

1.3.3. A brief overview of muscle protein turnover 8

1.3.3.1. Protein requirements for the maintenance of muscle 9

1.4. THE UK AGEING POPULATION 10

1.5. BODY COMPOSITION CHANGES WITH AGE 12

1.5.1. Alterations in muscle protein turnover with age 13

1.6. SARCOPENIA 14

1.6.1. Sarcopenia: brief overview 14

1.6.2. The Evolution of Definitions of Sarcopenia 15

1.6.2.1. Assessment of Muscle Mass in Sarcopenia 19

1.6.2.2. Assessment of muscle strength and function in Sarcopenia 20

1.6.3. Countermeasures to Sarcopenia 21

1.6.3.1. Resistance exercise in older adults 21

1.7. METHODS FOR ASSESSMENT OF MUSCLE MASS 22

1.7.1. Magnetic Resonance Imaging 22

1.7.2. Computed-Tomography 23

1.7.3. Dual-energy X-Ray Absorptiometry 24

1.7.4. Ultrasound 25

1.8. METHODS USED FOR THE ASSESSMENT OF MUSCLE METABOLISM 26

1.8.1. What is a stable isotope? 26

1.8.2. General introduction to mass spectrometry 28

1.8.3. Gas Chromatography-Pyrolysis-Isotope Ratio Mass Spectrometry 29

1.8.4. Liquid Chromatography Mass Spectrometry 30

1.8.5. TC- EA- IRMS 31

1.9. INTRAVENOUS INFUSION AND STABLE ISOTOPE APPROACHES TO MEASURE MUSCLE PROTEIN SYNTHESIS AND MUSCLE PROTEIN BREAKDOWN 31

1.9.1. Intravenous infusion of stable isotope labelled tracer; the primed constant infusion method 31

1.9.1.1. Primed constant infusion 32

1.9.2. Assessment of muscle protein synthesis using intravenous tracer infusion methods. 34

1.9.2.1. The arteriovenous balance method to measure MPS 34

1.9.2.2. Direct incorporation-precursor product approach to measure muscle protein synthesis. 35

1.9.3. Approaches to measure muscle protein breakdown using the intravenous infusion technique 37

1.9.3.1. AV Balance 37

1.9.3.2. Fractional Breakdown Rate 38

1.10. ORAL STABLE ISOTOPE TRACER APPROACHES TO MEASURE MUSCLE PROTEIN SYNTHESIS AND MUSCLE PROTEIN BREAKDOWN 39

1.10.1. Oral tracer provisions for muscle protein synthesis or breakdown measures . 39

1.10.1.1. The flooding dose 39

1.10.1.2. Use of D_2O 40

1.10.2. Approaches used to measure muscle protein breakdown from oral tracer provision 43

1.10.2.1. 3-Methylhistidine 43

1.11. ORAL STABLE ISOTOPE TRACER APPROACH TO MEASURE MUSCLE MASS USING LABELLED CREATINE 44

1.11.1.1. Creatine Metabolism 45

1.11.1.2. Measurement of muscle mass using stable-isotopically labelled creatine 47

1.12. SUMMARY 47

1.13. AIMS AND OBJECTIVES 49

2. GENERAL METHODS 50

2.1. SUBJECT CHARACTERISTICS AND ETHICS 50

2.2. STUDY DESIGN 51

2.2.1. Study Protocol 51

2.3. BASELINE MUSCLE FUNCTION TESTS 52

2.3.1. Short Physical Performance Battery Test 52

2.3.2. Handgrip Strength 54

2.3.3. Maximal Voluntary Contraction 54

2.3.4. Leg Strength 54

2.3.5. Leg Power 55

2.4. SAMPLE PROCESSING AND STORAGE 55

2.5. MUSCLE MASS ANALYSES USING D_3-CREATINE 55

2.5.1. Determination of Creatine Concentration and Enrichment in Urine Samples . 55

2.6. MUSCLE PROTEIN SYNTHESIS ANALYSES USING D_2O 57

2.6.1. Measurement of Body Water Enrichment 57

2.6.2. Preparation of Muscle for Measurement of Deuterium Enrichment 57

2.7. MUSCLE PROTEIN BREAKDOWN ANALYSES USING D_3-3-METHYLHISTIDINE 59

2.8. MUSCLE ARCHITECTURE MEASUREMENTS 60

2.9. DUAL-ENERGY X-RAY ABSORPTIOMETRY 61

3. EXPLORING THE CHARACTERISTICS OF AGEING IN RESEARCH PARTICIPANTS AND THE VARIABILITY IN SARCOPENIA IDENTIFICATION 62

3.1. INTRODUCTION 63

3.2. METHODS 64

3.2.1. Subject Characteristics and Protocol 64

3.2.2. Assessment of Body Composition, and Functional Assessment 66

3.2.3. Calculation of muscle mass cut-off indices 67

3.2.4. Statistical Analyses 67

3.3. RESULTS 68

3.3.1. Subject Characteristics 68

3.3.2. Blood Chemistry and QRisk3 69

3.3.3. Body composition changes with age 69

3.3.4. Correlation of Bone Indices and measures of strength. 70

3.3.5. Effect of Age and Gender on muscle mass 70

3.3.6. Effect Age and Gender on muscle strength and function 71

3.3.6.1. Application of existing muscle mass cut-offs to define Sarcopenia in our cohorts . 73

3.3.7. Application of different handgrip strength cut-offs to our cohorts 77

3.3.8. Application of different functional physical performance cut-offs 78

3.4. DISCUSSION 80

4. DEVELOPMENT OF COMBINED MINIMALLY INVASIVE STABLE ISOTOPE APPROACHES TO IDENTIFY ANY RELATIONSHIPS AND SIMULTANEOUSLY MEASURE MUSCLE PROTEIN TURNOVER AND MUSCLE MASS 91

4.1. INTRODUCTION 92

4.2. METHODS 94

4.2.1. Development of the D_3-Creatine method 94

4.2.1.1. Dosing and Sampling 94

4.2.1.2. Preparation of samples 94

4.2.1.3. LC conditions 95

4.2.1.4. MS conditions 96

4.2.1.5. Calculation of muscle mass 97

4.2.1.6. Statistical Analysis 97

4.2.2. Development of the D_3-3-methylhistidine method 97

4.2.2.1. Dosing and Sampling 97

4.2.2.2. Preparation of samples 98

4.2.2.3. LC -conditions 98

4.2.2.4. MS conditions 98

4.2.2.5. Calculation of muscle protein breakdown 99

4.2.2.6. Statistical analysis 99

4.2.3. Calculation of Muscle Protein Fractional Synthetic Rate using D_2O 100

4.2.3.1. Dosing and Sampling 100

4.2.3.2. Preparation of Samples 101

4.2.3.3. TC-EA conditions 101

4.2.3.4. Calculation of Fractional Synthetic Rate 102

4.2.4. Combination of the three approaches 103

4.2.5. Quantification of MPB in intervention studies using D_3-3-methylhistidine ... 104

4.3. RESULTS 105

4.3.1. Determination of D_3-creatine spill over 105

4.3.2. D_3-creatinine enrichment 107

4.3.3. D_3-creatine muscle mass quantification and correlation to ALBM from DXA 107

4.3.4. D_3-creatine vs DXA 108

4.3.5. D_3-3-methylhistidine enrichment decay 110

4.3.6. Determination of rates of MPB by D_3-3-methylhistidine 110

4.3.7. Body water enrichment 111

4.3.8. Myofibrillar Fractional Synthetic Rates 112

4.3.9. Application of the D_3-3-methylhistidine approach to measure MPB before and after an intervention 113

4.4. DISCUSSION 114

5. APPLICATION OF THE COMBINED ORAL STABLE ISOTOPE TRACER APPROACH TO ASSESS MUSCLE PROTEIN SYNTHESIS, MUSCLE PROTEIN BREAKDOWN AND MUSCLE MASS IN A LARGE COHORT OF OLDER ADULTS 121

5.1. INTRODUCTION 122

5.2. METHODS 125

5.2.1. Subjects and protocol 125

5.2.2. Dosing and Sampling 125

5.2.3. Statistical Analysis 127

5.3. RESULTS 128

5.3.1. Subject Characteristics *128*

5.3.1. Muscle mass using D_3-Creatine *129*

5.3.1.1. Labelled Creatine in Urine 129

5.3.1.2. D_3-Creatinine Enrichment in urine over time 130

5.3.2. Muscle architecture *131*

5.3.3. Myofibrillar fractional synthetic rate *131*

5.3.3.1. Body water deuterium enrichment 131

5.3.3.2. Myofibrillar fractional synthetic rates 132

5.3.4. Whole-body muscle protein breakdown rate using D_3-3MH *133*

5.3.4.1. Dilution of D_3-3-methylhistidine 133

5.3.4.2. Rates of whole-body muscle protein breakdown 134

5.3.5. Comparison of D_3-Creatine method vs DXA *135*

5.3.1. Correlation between muscle mass measured by D3-Creatine and muscle thickness by ultrasound *137*

5.3.2. Relationships between muscle mass and muscle strength and power *138*

5.3.3. Identifying relationships between muscle mass and muscle protein turnover (MPS & MPB) *142*

5.3.4. Relationship between MPS and MPB and MPS/MPB with muscle strength *146*

5.4. DISCUSSION 147

6. A PRACTICAL HOME-BASED INTERVENTION OF A 4-WEEK RESISTANCE EXERCISE PROGRAMME AIMED AT ENHANCING MUSCLE MASS AND FUNCTION IN OLDER ADULTS: A PILOT STUDY **163**

ABSTRACT: **164**

6.1. INTRODUCTION 165

6.2. MATERIALS AND METHODS 167

6.2.1. Recruitment of study participants and ethical approval *167*

6.2.2. Study procedures *167*

6.2.3. Home-based exercise training programme (HBET) *169*

6.2.4. Measurement of whole-body muscle mass using D_3-creatine 170

6.2.5. Statistical analysis 171

6.3. RESULTS 171

6.3.1. Compliance, body weight and cardiovascular parameters 171

6.3.2. Muscle function 172

6.3.3. Muscle architecture 174

6.3.4. Whole-body muscle mass measured by D_3-Creatine 174

6.4. DISCUSSION 175

7. OVERALL DISCUSSION 179

Table of Figures

Figure 1.2.1.1 Muscle structure 2

Figure 1.2.1.1 Myofibril and Sarcomere structure when relaxed and contracted 3

Figure 1.3.3.1 Population estimates of the very old (90+ years) in the United Kingdom from mid-2002 to mid-2018. Estimates used have been rounded to the nearest 10. Data from Office for National Statistics, 2019. 11

Figure 1.3.3.2 Data collected over a number of years, outlining life expectancy and healthy life expectancy. Data from Office for National Statistics, 2019. 11

Figure 1.6.2.1 A comparison of the definitions of sarcopenia developed by the European Working Group on Sarcopenia in Older People (EWGSOP), including their updated version (EWGSOP2) and by the International Working Group on Sarcopenia (IWGS) and the Foundation for the National Institutes of Health (FNIH). 18

Figure 1.8.2.1 Basic depiction of sample analysis by mass spectrometry 28

Figure 1.9.2.1 A representation of the 3-pool arteriovenous balance method where A represents the arterial pool, V the venous amino acid pool and M the intramuscular amino acid pool. The direction of the arrows is representative of the flow of the amino acid. 35

Figure 1.9.3.1 Schematic of movement of labelled (dotted lines) and unlabelled (filled lines) amino acids, from the arterial pool (A) to the intramuscular AA pool (M) as well as to and from the muscle (MP). Adapted from Zhang et al. 1996 & Volpi et al. 2001 38

Figure 1.10.2.1 Cr biosynthesis, transport and excretion (Adapted from Wyss & Kaddurah-Daouk, 2000) 46

Figure 2.2.1.1 Group 1 Study Protocol 52

Figure 2.6.2.1 Example of how pennation angle would be determined from a m. vastus lateralis muscle ultrasound image. 60

Figure 2.6.2.1 Example of body positioning for DXA 61

Figure 3.3.4.1 Correlations between (A) total BMC and handgrip strength, (B) total BMC and single-leg extension 1RM and C) single leg-extension 1RM and total BMD, all in the older (>65 years) females. 70

FIGURE 3.3.5.1 YOUNG (18-35Y) VS. OLD (>65Y) LEAN MUSCLE MASS ESTIMATED BY DXA AND SEPARATED MALE AND FEMALE. ** DENOTES P= 0.00991 AND * WHERE P=0.023 71

FIGURE 3.3.5.2 DECLINE OF MUSCLE MASS WITH AGE WITHIN THE OLDER COHORT (>65Y) 71

FIGURE 3.3.6.1 A) NEGATIVE REGRESSION OF 1-RM UNILATERAL LEG EXTENSION STRENGTH WITH AGE. B) A SIGNIFICANT POSITIVE CORRELATION OF MUSCLE MASS AND 1-RM UNILATERAL LEG EXTENSION FOR BOTH MALES AND FEMALES. **** DENOTES P<0.0001 AND * WHERE P=0.017. 72

FIGURE 3.3.6.2 A) CORRELATIONS BETWEEN 1-RM UNILATERAL LEG EXTENSION B) HANDGRIP STRENGTH C) SINGLE LEG MVC D) GAIT SPEED AND AGE IN OLDER (65+) MALES AND FEMALES. 73

FIGURE 3.3.6.3 THE DIFFERENCE OF APPENDICULAR LEAN MASS (ALM) (A AND B), APPENDICULAR SKELETAL MUSCLE INDEX (ASMI: I.E. ALM/HT^2) (C AND D) AND APPENDICULAR LEAN MASS CORRECTED FOR BMI (E AND F) BETWEEN YOUNG AND OLD, MALE AND FEMALE. **** DENOTES P<0.0001 76

FIGURE 3.3.7.1 THE UPDATED EWGSOP2 CUT-OFF POINT VS THE ORIGINAL EWGSOP CUT-OFF IN FEMALES A) AND MALES B). 78

FIGURE 3.3.8.1 SCORING FOR HANDGRIP STRENGTH VS GAIT SPEED FOR OLDER MALES (A) AND OLDER FEMALES (B) 79

FIGURE 4.2.1.1 EXAMPLE CHROMATOGRAMS FOR THE DETERMINATION OF ^{12}CREATINE, ^{13}CREATINE AND D_3CREATINE 96

FIGURE 4.2.2.1 EXAMPLE CHROMATOGRAPHS OF 3-METHYLHISTIDINE AND D_3-3-METHYLHISTIDINE 99

FIGURE 4.2.3.1 EXAMPLE TRACE FOR TWO REFERENCE PEAKS AND A SAMPLE WITH THE RED LINE IDENTIFYING MASS 2 AND THE GREEN LINE MASS 3. 102

FIGURE 4.2.4.1 PROTOCOL FOR COMBINED TRACER APPROACH 103

FIGURE 4.3.1.1 URINE D_3-CREATINE TO UNLABELLED CREATINE RATIO AT TIME POINTS 0, 24, 48 AND 72H. . 106

FIGURE 4.3.1.2 PERCENTAGE OF DOSE EXCRETED IN THE 0-24 H URINE IN OLDER MALES OF THIS STUDY (A) AND OLDER MALES FROM THE STUDY BY CLARK ET AL. (2014) (B). VALUES DISPLAYED AS MEDIAN AND MIN-MAX. 106

FIGURE 4.3.2.1 D_3-CREATININE ENRICHMENT RATIO CORRECTED TO BASELINE AT 0, 24-POOL, 48 AND 72H. 107

FIGURE 4.3.4.1 MUSCLE MASS MEASURED BY D_3-CR VS A) TOTAL LEAN BODY MASS (LBM) AND B) APPENDICULAR LEAN BODY MASS (ALBM) BY DXA. 109

Figure 4.3.5.1 Mean D_3-3-methylhistidine enrichment in plasma from 24-30h post tracer ingestion (Mean ± 95%CI) 110

Figure 4.3.6.1 Example determination of the rate of muscle protein breakdown (k) from ln transformed plasma D3-3-methylhistidine enrichment ratio 111

Figure 4.3.6.2 Mean rate of whole-body muscle protein breakdown determined by D_3-3-methylhistidine in 10 older males (Mean ± 95%CI).....111

Figure 4.3.7.1 Mean body water enrichment of D_2O at 2, 12, 24, 48 and 72h (Mean ± 95%CI) ...112

Figure 4.3.8.1 Mean rate of myofibrillar fractional synthetic rate (FSR). Mean and 95% CI. 112

Figure 4.3.9.1 The effect of 7-day bed- rest on whole-body MPB rates measured by D_3-3MH (n=12, mean ± SD) 113

Figure 4.3.9.2 Whole-body MPB decay rate at baseline, week 3 and week 6 of the placebo and testosterone groups. (Mean ± SEM, Placebo: n=9, Testosterone: n=9)(Gharahdaghi et al., 2019).....114

Figure 5.3.1.1 Corrected ratio of D_3-Creatine/Creatine excreted in the urine for samples collected at Baseline (0h), 24h pool, 48 and 72h 129

Figure 5.3.1.2. Percentage dose not absorbed by the body in 0-24 h urine collection sample. Values displayed are min to max plots.....130

Figure 5.3.1.3 Ratio of labelled creatinine (D_3-creatinine) to unlabelled creatinine, corrected to the baseline sample (0h) for 24,48 and 72h urine samples in both males and females. Mean ± 95% CI. 131

Figure 5.3.3.1 A) Deuterium enrichment of the body water, measured in APE from saliva samples at baseline, 2, 12, 24, 48 and 72h post- deuterium oxide ingestion. B) Mean deuterium enrichment over the 72h time period in males and females. **** denotes p<0.0001.....132

Figure 5.3.3.2 Myofibrillar fractional synthetic rates for males and females from the Abbeyfield study. Mean and 95% CI.....133

Figure 5.3.4.1 Decay curves of D_3-3-methylhistidine in the plasma by endogenous 3-methylhistidine release for both males (n=21) and females (n=16). Mean and 95% CI. 134

FIGURE 5.3.5.1 A CORRELATION BETWEEN MUSCLE MASS MEASURED BY D_3-CREATINE METHOD WITH LEAN BODY MASS (LBM) MEASURED BY DXA AND APPENDICULAR LEAN BODY MASS (ALBM) WHEN DATA ARE COMBINED (A) AND SEPARATED BY GENDER (B).....136

FIGURE 5.3.1.1 CORRELATION BETWEEN MUSCLE MASS MEASURED BY D_3-CREATINE AND M. VASTUS LATERALIS THICKNESS MEASURED BY ULTRASOUND SEPARATED BY MALE AND FEMALE (A) AND COMBINED (B). 137

FIGURE 5.3.1.2 CORRELATION BETWEEN APPENDICULAR LEAN BODY MASS AND M. VASTUS LATERALIS THICKNESS MEASURED BY ULTRASOUND SEPARATED BY MALE AND FEMALE (A) AND COMBINED (B). 138

FIGURE 5.3.2.1 CORRELATIONS BETWEEN MUSCLE MASS MEASURED BY D_3-CREATINE WITH (A) SINGLE-LEG 1RM LEG EXTENSION, (B) SINGLE LEG MAXIMUM VOLUNTARY CONTRACTION, (C) HANDGRIP STRENGTH AND (D) LEG POWER FOR BOTH MEN AND WOMEN COMBINED. 140

FIGURE 5.3.2.2 CORRELATIONS BETWEEN MUSCLE MASS MEASURED BY D_3-CREATINE WITH (A) SINGLE-LEG 1RM LEG EXTENSION, (B) SINGLE LEG MAXIMUM VOLUNTARY CONTRACTION, (C) HANDGRIP STRENGTH AND (D) LEG POWER FOR BOTH MEN AND WOMEN.....140

FIGURE 5.3.2.3 CORRELATIONS BETWEEN APPENDICULAR LEAN BODY MASS WITH (A) SINGLE-LEG 1RM LEG EXTENSION, (B) SINGLE LEG MAXIMUM VOLUNTARY CONTRACTION, (C) HANDGRIP STRENGTH AND (D) LEG POWER FOR BOTH MEN AND WOMEN COMBINED. 141

FIGURE 5.3.2.4 CORRELATIONS BETWEEN APPENDICULAR LEAN BODY MASS WITH (A) SINGLE-LEG 1RM LEG EXTENSION, (B) SINGLE LEG MAXIMUM VOLUNTARY CONTRACTION, (C) HANDGRIP STRENGTH AND (D) LEG POWER FOR BOTH MEN AND WOMEN. 141

FIGURE 5.3.3.1 LINEAR REGRESSION OF DXA LEAN BODY MASS (LBM), APPENDICULAR LEAN BODY MASS (ALBM) MEASURES WITH FRACTIONAL SYNTHETIC RATE (FSR) OF THE M. VASTUS LATERALIS WHERE DATA ARE COMBINED IN A) AND SEPARATED BY GENDER IN B). LINEAR REGRESSION BETWEEN FSR AND MUSCLE MASS MEASURED USING THE D3-CREATINE METHOD WITH DATA COMBINED (C) AND SEPARATED BY GENDER (D). 143

FIGURE 5.3.3.2 LINEAR REGRESSION OF MUSCLE MASS MEASURED BY D_3-CR AND WHOLE-BODY MPB WITH DATA COMBINED (A) AND SEPARATED BY GENDER (B).....144

FIGURE 5.3.3.3 CORRELATIONS BETWEEN NET BALANCE AND MUSCLE MASS MEASURED BY D_3-CREATINE (A) AND DXA (B).....145

FIGURE 5.3.4.1 NO SIGNIFICANT CORRELATION BETWEEN MYOFIBRILLAR FSR AND RATE OF MPB REPRESENTED BY THE RATE OF D_3-3MH DILUTION 146

FIGURE 6.2.3.1 **STUDY SCHEMATIC DISPLAYING THE TWO VISITS BEFORE AND AFTER HOME-BASED EXERCISE TRAINING (HBET).** BL: BASELINE URINE SAMPLE; HR: RESTING HEART RATE; BP: RESTING BLOOD PRESSURE; SPPBT: SHORT PHYSICAL PERFORMANCE BATTERY TEST; US: LEG ULTRASOUND; MVC: MAXIMUM VOLUNTARY CONTRACTION; 1-RM: LEG EXTENSION 1-REPETITION MAXIMUM; D_3-CR: D_3-CREATINE FOR WHOLE-BODY MUSCLE MASS ASSESSMENT; 48 AND 72H REPRESENT THE TIME OF URINE SAMPLES POST-TRACER CONSUMPTION. 170

FIGURE 6.3.2.1 **MUSCLE (LEG) FUNCTION ASSESSMENTS, BEFORE AND AFTER HOME-BASED EXERCISE TRAINING (HBET).** A) 1-RM LEG EXTENSION (45.7±5.9 VS. 49.6±6.0 KG, P=0.08), B) LEG POWER VIA NOTTINGHAM POWER RIG (276.7±38.5 VS. 323.4±43.4 W), C) MVC AT 60° (154.2±18.4 VS. 168.8±15.2 NM), D) MVC AT 90° (152.1±10.5 VS. 159.1±11.4 NM), E) SEATED LEG FLEXION AT 60°/SEC (74.7±8.3 VS. 80.8±8.6 NM), F) SEATED LEG EXTENSION AT 60°/SEC (114.9±10.2 VS. 123.8±9.4 NM). BASELINE VS. POST-HBET (MEAN ± SEM), N=12, *=P<0.05; **=P<0.01. MVC: MAXIMAL VOLUNTARY CONTRACTION. 173

FIGURE 6.3.4.1 **A) QUADRICEPS CROSS-SECTIONAL AREA** (CSA; 57.5±5.4 VS. 59.0 ± 5.3 MM) WITH INDIVIDUAL DATA POINTS OUTLINED IN B); **C) M. VASTUS LATERALIS (VL) THICKNESS** (25.0±1.6 VS. 25.5±1.7 MM, P=0.08 WITH INDIVIDUAL DATA POINTS OUTLINED IN D); **E) WHOLE-BODY MUSCLE MASS, BEFORE AND AFTER HOME-BASED EXERCISE TRAINING (HBET)** (23.9±2.1 VS. 22.8±2.2 KG) WITH INDIVIDUAL DATA POINTS OUTLINED IN F). (MEAN ± SEM), *=P<0.05, BASELINE VS. POST-HBET. 175

List of Tables

TABLE 1.6-1 GRADING STRUCTURE FOR THE SEVERITY OF SARCOPENIA SUGGESTED BY THE EWGSOP 16

TABLE 2-1 AN OVERVIEW OF THE SCORING SYSTEM USED FOR THE SPPBT. 53

TABLE 3.3.1-1 SUBJECT CHARACTERISTICS. VALUES DISPLAYED AS MEAN ± SD. STATISTICALLY SIGNIFICANT DIFFERENCE TO THE YOUNG MALE GROUP IS REPRESENTED BY A, B- YOUNG FEMALE GROUP, C-OLD MALE GROUP AND D-OLD FEMALE GROUP. (TOTAL ARMS LEAN MASS WAS MISSING FOR SOME INDIVIDUALS THEREBY REDUCING THE NUMBER ALBM COULD BE MEASURED FOR). 68

TABLE 3.3.6-1 APPLICATION OF DIFFERENT SARCOPENIA CUT-OFF VALUES FOR MUSCLE MASS PROVIDED BY THE EWGSOP, EWGSOP2, IWGS AND FNIH TO THE YOUNG AND OLD PARTICIPANTS. ALM- APPENDICULAR LEAN MASS, ALM/HT^2- APPENDICULAR LEAN MASS (KG) DIVIDED BY HEIGHT (IN M) SQUARED. 74

TABLE 4-1 CREATINE POOL SIZE AND MUSCLE MASS MEASURES FOR THE OLDER MALES (65Y+). MEAN ±SD. . 108

TABLE 5.3.1-1 SUBJECT CHARACTERISTICS. P VALUE FROM DIFFERENCES OBSERVED BETWEEN MALES AND FEMALES. NS- DENOTES NO SIGNIFICANT DIFFERENCE BETWEEN MALE AND FEMALE (P>0.05). 128

TABLE 5.3.2-1 MUSCLE ARCHITECTURE MEASUREMENTS. VALUES DISPLAYED AS MEAN ± SD. 131

Abbreviations

AA	Amino acids
ADP	Adenosine diphosphate
AGAT	L-Arginine:glycine amidinotransferase
ALBM	Appendicular lean body mass
APE	Atom percent excess
ASMI	Appendicular skeletal muscle index
ATP	Adenosine triphosphate
BCAA	Branched chain amino acid
BIA	Bioelectrical impedance analyser
BMI	Body mass index
CK	Creatine kinase
Cr	Creatine
Crn	Creatinine
CSA	Cross sectional area
CT	Computed tomography
DEXA	Dual-energy X-ray absorptiometry
D_2O	Deuterium oxide
EAA	Essential amino acids
EWGSOP	European working group on Sarcopenia in older people
FSR	Fractional synthetic rate
GC-MS	Gas chromatography mass spectrometry
HBET	Home-based exercise training
HILIC	Hydrophilic interaction liquid chromatography

IRMS	Isotope ratio mass spectrometry
LC-MS	Liquid chromatography mass spectrometry
LBM	Lean body mass
MPB	Muscle protein breakdown
MPS	Muscle protein synthesis
MRI	Magnetic resonance imaging
MT	Muscle thickness
MVC	Maximum voluntary contraction
NEAA	Non-essential amino acid
PCA	Perchloric acid
PCr	Phosphocreatine
SMI	Skeletal muscle index
TC-EA	High temperature conversion elemental analyser
VL	Vastus lateralis
1-RM	1-repetition maximum
3MH	3-methylhistidine

1. Introduction

1.1. The Importance of Skeletal Muscle in Health, Ageing and Disease

Skeletal muscle is a highly metabolically active tissue, accounting for approximately 50% of total body mass (Koopman, 2011). In addition to its' primary role in locomotion and posture, skeletal muscle is a crucial tissue that contributes to many metabolic functions; to the extent that they stimulate heat production for the maintenance of core body temperature (Zierath and Hawley, 2004), are an energy store (Rennie *et al.*, 1982) and provide a vital role in the maintenance of whole-body metabolic health by acting as a site for glucose disposal and as the largest protein store in the body (Rudrappa *et al.*, 2016). Moreover, skeletal muscle accounts for 30-50% of total protein turnover within the body (Gasier *et al.*, 2009; Gasier, Fluckey and Previs, 2010; Frontera and Ochala, 2015), emphasising the highly metabolic activity of the tissue (Gasier, Fluckey and Previs, 2010). Therefore, unsurprisingly, the loss of skeletal muscle mass is associated with numerous diseases e.g. diabetes (Severinsen *et al.*, 2007), AIDs (Sheikh *et al.*, 1999), cancer (Murphy *et al.*, 2011) as well as disuse atrophy and also ageing (i.e. sarcopenia) (Atherton *et al.*, 2016).

Consequently, furthering our understanding of the factors that regulate muscle metabolism e.g. nutritional/ exercise/ pharmacological as well as the techniques in which we can quantify these factors is vital in improving quality of life of people with any

condition associated with muscle-wasting. The latter sections of the introduction will display the vast number of techniques that can be used to measure muscle metabolism. However, first it is useful to understand the structure and mechanics of muscle to comprehend how it is then metabolised.

1.2. Skeletal Muscle Structure

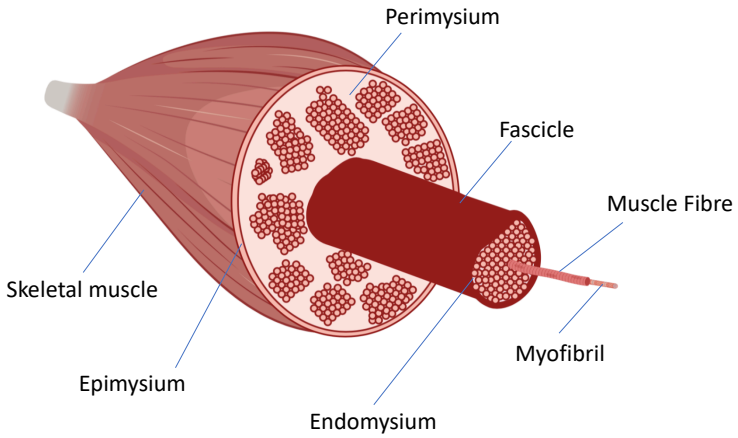


Figure 1.2.1.1 Muscle structure

1.2.1. Structure overview

It has been well established that the main role of skeletal muscle is to aid locomotion and posture. In order for this to be achieved, skeletal muscles are attached to bone by connective tissue known as tendons (Kjær *et al.*, 2006; Frontera and Ochala, 2015). For movement to occur, one end of the muscle is attached to an immobile bone (also known as the *point of origin*), whilst the other is attached to a mobile bone (known as *point of insertion*). The whole of the

muscle is surrounded by a strong fibrous layer of connective tissue known as *fascia.* A thinner layer of connective tissue, termed the *epimysium,* lies just underneath the fascia (See Figure 1.2.1.1). The epimysium encloses bundles of 10 to 100 muscle fibres, termed *fascicles* (Gillies and Lieber, 2011). The connective tissue surrounding the fascicles is known as the *perimysium.* The muscle fibres within the fascicles are encompassed by another layer of connective tissue known as the *endomysium* (Broek, Grefte and Von Den Hoff, 2010).

The skeletal muscle fibres are highly specialised postmitotic, multinucleated cells (Schmalbruch and Lewis, 2000; Chargé and Rudnicki, 2004). The myonuclei can be found on the periphery of these long, cylindrical cells (Chargé and Rudnicki, 2004; Relaix and Zammit, 2012). The cytoplasm of the muscle cell is known as the *sarcoplasm* containing intracellular fluid, proteins, enzymes and sub-cellular organelles such as mitochondria.

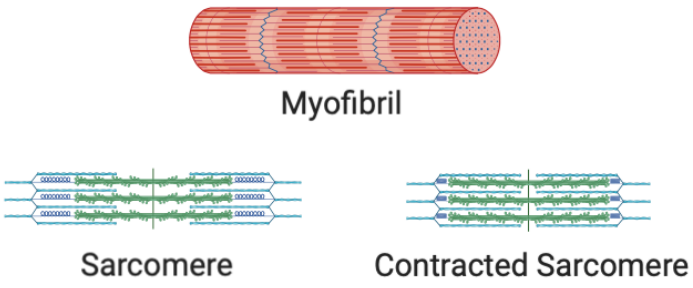


Figure 1.2.1.1 Myofibril and Sarcomere structure when relaxed and contracted

Additionally, the sarcoplasm contains myofibrils that are composed of two major contractile protein actin (main component of the thin filaments) and myosin (thick filaments). These proteins are arranged in sarcomeres, which are stacked in repeated units. The z-lines at the

end of each sarcomere provide the striated appearance to skeletal muscle. The structure of the sarcomeres and interaction between the actin and myosin filaments is key in allowing a muscle contraction to occur. More detail on muscle contraction can be found in the following review (Frontera and Ochala, 2015). The degradation of the actomyosin structures can be measured to assess whole-body protein breakdown and will be discussed later on in detail.

1.2.2.Muscle Fibres

Following the basic overview of skeletal muscle structure, it is important to understand that different whole muscles will comprise of different fibre-type compositions which will reflect on the muscle's performance and protein kinetics. Human muscle contain 3 muscle fibre-types; type I, type IIa and IIb (Broek, Grefte and Von Den Hoff, 2010). Type I fibres are also known as slow-twitch or oxidative fibres. These slow-fatiguing fibres have a low excitation threshold as they are innervated by small α-motor neurons (Burke and Edgerton, 1975). Type I fibres can appear redder in colour than type II fibres, due to their high myoglobin content (Eisenberg, 1983; Zierath and Hawley, 2004; Frontera and Ochala, 2015). In contrast, type II fibres are known as fast-twitch fibres due to their faster shortening velocity that correlates with greater myosin ATPase activity (Bárány, 1967; Scott, Stevens and Binder-macleod, 2001). Type IIb fibres are fast-fatiguing muscle fibres (Eisenberg, 1983). Alternatively, type IIa fibres are known to be fast, fatigue resistant fibres whose properties are considered as an intermediate between type I and type IIb fibres (Eisenberg, 1983). Based on the various characteristics of each fibre

type, the composition of fibres within a muscle group will determine the performance of aerobic or anaerobic activities for the muscle (Zierath and Hawley, 2004). In cases of sarcopenia, a greater atrophy of type II fibres than of the slow-twitch type I fibres has been identified (Lexell, Taylor and Sj, 1988). This pattern of fibre atrophy is also observed in cancer cachexia (Tisdale, 2009). How muscle mass is regulated will be discussed in the following section.

1.3. Muscle Protein Turnover

1.3.1. Muscle Protein Synthesis

The pathway for protein synthesis involves the transcription of DNA to form messenger RNA (mRNA) and then translating this message to form a specific protein (Kimball, 2013). The translation, or decoding, of the mRNA structure by ribosomes can be divided into three distinct processes: initiation, elongation and termination (Proud, 2009). In brief, the ribosome acts as an enzyme in the decoding of mRNA via the attachment of transfer RNA. In humans, the different factors involved in these steps are known as eukaryotic initiation, elongation and release factors (eIF's, eEF's and eRF's).

The initiation step of translation involves the assembly of the ribosomal complex at the start codon of the mRNA structure, regulated by complex interactions between initiation factors and signalling proteins (Drummond *et al.*, 2009). In eukaryotes, the most common start codon is AUG. The 80S ribosome is separated into 60S and 40S subunits eIF3 and eIF1A. eIF3 binds to the 40S subunit to create a 43S pre-initiation complex. Initiator metionyl-tRNA (met-$tRNA_i$) binds to the eukaryotic initiation factor eIF2. To aid translation

initiation, several eIF4 factors help the ribosomal complex to bind to the mRNA. The eIF4e guides the 5'end of mRNA into the 43S pre-initiation complex (Proud, 2007). The ribosome continues along the mRNA structure until the met-$tRNA_i$ identifies the start codon. Once found, the 60S ribosomal subunit is recruited by eIFs to create the 80S complex. The process of elongation and subsequently polypeptide/protein synthesis begins.

During elongation eEFs recruit tRNA to the corresponding mRNA codons. The two main factors involved in this process are eEF1A and eEF2. The process involves the binding of active tRNA (i.e. with the amino acid), the formation of a peptide bond and then releasing the inactive tRNA. This process of elongation will continue along the mRNA structure until the 80S complex reaches the stop codon (either UAA, UAG or UGA)(Welch, Wang and Peltz, 2000). This step known as termination is controlled by the eRFs and prohibits the ribosome from continuing polypeptide formation (Welch, Wang and Peltz, 2000).

1.3.2.Muscle Protein Breakdown

There are 4 main pathways for protein degradation to occur; lysosomal-autophagy, the ubiquitin-proteasome system, cysteine-dependent proteases (caspases) and Ca^{2+}- dependent proteases (calpains) (Murton, Constantin and Greenhaff, 2008). The ubiquitin-proteasome pathway has been considered the main catabolic system for the breakdown of contractile proteins and therefore myofiber atrophy (Sandri, 2010). Initially, E1 (a ubiquitin-activating enzyme)

binds to free ubiquitin via thiolesterification in an ATP-dependent reaction (Murton, Constantin and Greenhaff, 2008). Subsequently, through transthioesterification the ubiquitin is then transferred to a ubiquitin-conjugating enzyme (E2) (Tipton, Hamilton and Gallagher, 2018). The ubiquitin is finally transferred onto the target protein via an E3 enzyme (Gomes *et al.*, 2001). This process is repeated until a polyubiquitin chain of at least 4 ubiquitin monomers are attached to the target protein (Tipton, Hamilton and Gallagher, 2018). This signals the 26S proteasome to begin proteolysis. Two genes identified as key regulators of this pathway have been Muscle Atrophy F-box (*MAFbx)* and Muscle RNG Finger 1 (*MuRF1)*, as both are upregulated during muscle atrophy (Bodine *et al.*, 2001). A more detailed review on the ubiquitin-proteasome system can be found in the following references (Hershko and Ciechanover, 1998; Murton, Constantin and Greenhaff, 2008).

The autophagy-lysosome pathway is the other main proteolytic pathway involved in the removal of many intracellular proteins and organelles (Masiero *et al.*, 2009). Autophagy involves the formation of a double-membrane vacuole known as an autophagosome which engulfs parts of the cytoplasm and intracellular organelles (Mammucari *et al.*, 2007). Subsequently, the autophagosome fuses with the lysosome for degradation of contents to their components e.g. proteins into amino acids (Lum, DeBerardinis and Thompson, 2005). One of the transcription factors known to regulate this pathway is Forkhead box O (FoxO) through inhibition of the Akt/PKB pathway.

1.3.3.A brief overview of muscle protein turnover

It is important to highlight that muscle is an extremely plastic tissue that can adapt to different pathophysiological, mechanical and metabolic changes (Marcotte, West and Baar, 2014). Muscle mass is regulated by the net balance between muscle protein synthesis (MPS) and muscle protein breakdown (MPB) (Rooyackers and Nair, 1997). In order to maintain muscle mass, both of these metabolic states exist in a dynamic equilibrium that is in constant flux depending on environmental factors such as nutrition and physical activity or hormones (S M Phillips, Glover and Rennie, 2009; Atherton and Smith, 2012).

In the postabsorptive, resting state, MPB will exceed MPS resulting in a negative protein balance (Rudrappa *et al.*, 2016). Rates of MPB and MPS in the postabsorptive state have been reported as ~0.08-0.11%/h (Phillips *et al.*, 1997, 1999) and ~0.02-0.095%/h (Smith *et al.*, 2013), respectively. This provides an overall negative net balance which if maintained, would result in muscle atrophy. However, a stimulus such as feeding increase the rates of MPS to a greater rate than MPB causing a positive net protein balance. During this time, any amino acids lost from the protein breakdown, can be replenished. Furthermore, amino acids that are deaminated and oxidised must be replaced as those from breakdown could be recycled. In terms of feeding response, the most effective stimulants are essential amino acids (EAAs) in particular, Leucine (one of the branched chain amino acids) has been identified as the most potent (Buse and Reid, 1975;

Fulks, Li and Goldberg, 1975; Smith *et al.*, 1992; Philip J Atherton, Smith, *et al.*, 2010). However, the effects of feeding are short-lived in terms of MPS, as rates of MPS will remain elevated for around 2h before returning to baseline (Bohé *et al.*, 2001; Rennie *et al.*, 2006). The muscle has an acquired "set-point" which has been termed the "muscle full effect" whereby the rate of MPS will not remain elevated despite a sufficient amount of freely available AAs (Philip J. Atherton *et al.*, 2010). Any excess AAs will be oxidised with remaining nitrogen being excreted as urea (Tarnopolsky, MacDougall and Atkinson, 1988).

1.3.3.1. *Protein requirements for the maintenance of muscle*

Given that protein turnover is dynamic and in a constant state of flux with significant amounts of urea excreted daily, these losses need to be replenished to maintain muscle mass. In addition, requirements may differ based upon protein turnover, existing protein intake, activity (athletes, power vs endurance) and gender.

Currently the recommended daily allowance of protein set by the World Health Organisation and Food and Agricultural Organisation of the United Nations is 0.8g/kg/day (Joint FAO/WHO/UNU Expert Consultation, on Protein in Human Nutrition (2002 : Geneva Switzerland) Food Agriculture Organization of the United Nations, World Health Organisation, United Nations University, 2007). However, the UK have a slightly lower recommended nutrient intake of 0.75g/kg/day (Committee on Medical Aspects of Food Policy, 1991), which could be problematic in negating sarcopenia.

Further states of negative net protein balance are acquired through injury/ trauma, sepsis (De Bandt and Cynober, 2006; Lang, Frost and Vary, 2007) and diseases (Anker and Sharma, 2002) such as cancer (Fearon, Voss and Hustead, 2006; Gallagher *et al.*, 2012; Vaughan, Martin and Lewandowski, 2013). Exercise increases rates of MPS yet, net protein turnover tends to remain negative as exercise also elevates rates of MPB (Kumar, Selby, *et al.*, 2009). A net positive balance can be achieved through the addition of appropriate protein feeding with exercise i.e. EAAs (Kumar, Atherton, *et al.*, 2009). Amongst this diurnal rhythm and constant flux, the overall myofibrillar muscle protein turnover rate in healthy young individuals is ~1.2-1.6%/day (Atherton and Smith, 2012).

1.4. The UK ageing population

The United Kingdom (UK) is experiencing an ageing population (See Figure 1.3.3.1), with estimates of 1 in 4 people to be aged 65 years and over by 2050 (Office of National Statistics, 2019). Moreover, life expectancy projected from the time of birth had been increasing, however, the number of years considered to be "healthy" are not as high (Greenhaff *et al.*, 2019; Office of National Statistics, 2019). Figure 1.3.3.2.

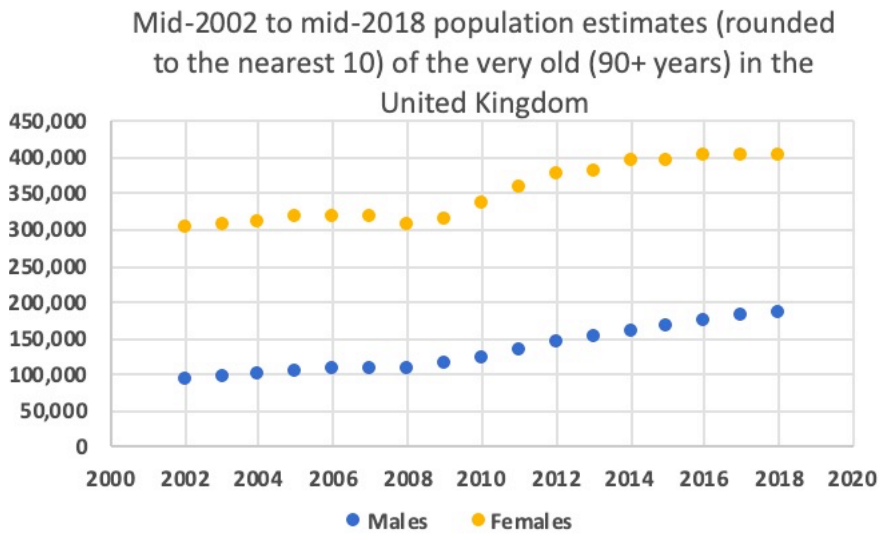


Figure 1.3.3.1 Population estimates of the very old (90+ years) in the United Kingdom from mid-2002 to mid-2018. Estimates used have been rounded to the nearest 10. Data from Office for National Statistics, 2019.

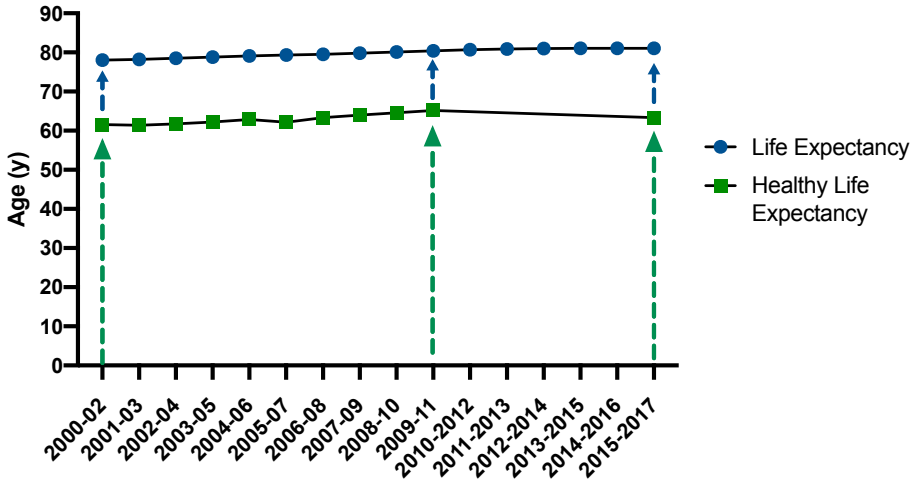


Figure 1.3.3.2 Data collected over a number of years, outlining life expectancy and healthy life expectancy. Data from Office for National Statistics, 2019.

With ageing, there is an increased risk of falls and fractures accompanied by loss of independence. Sarcopenia is a term used to describe progressive muscle atrophy associated with ageing in concordance with a loss of muscle function and strength (Cruz-Jentoft *et al.*, 2010; Narici and Maffulli, 2010). If loss of physical performance is present alongside loss of muscle mass and strength, this is defined as severe sarcopenia. With an increased awareness of this "geriatric

syndrome" (Cruz-Jentoft *et al.*, 2010; Chen *et al.*, 2014), sarcopenia was recognised as a condition that should be diagnosed in older people and was subsequently identified as an independent disease in the International Classification of Disease, 10th revision, Clinical Modification (ICD-10-CM) in 2016 (Anker, Morley and von Haehling, 2016). The accelerated loss of muscle mass has been recorded as 1%/ year from the age of 50 with a greater loss observed with subsequent decades (Goodpaster *et al.*, 2006; Keller and Engelhardt, 2013). A main cause of muscle loss is inactivity whether that is due to age-related immobility, bed-rest, trauma or diseases such as cancer (Stephens *et al.*, 2010; Morley *et al.*, 2011; Puthucheary *et al.*, 2013). In order to assess changes in muscle atrophy, studies use experimental means such as bedrest (Symons *et al.*, 2009), limb-cast immobilisation (Dideriksen *et al.*, 2020) and unilateral lower limb suspensions (Gustafsson *et al.*, 2010). Such studies may provide inside into the mechanisms of muscle atrophy that may relate with ageing. The loss of muscle strength with age (also referred to as "dynapenia") has been reported as 3-4%/ year in men and 2.5%/ year in women (Mitchell *et al.*, 2012). Sarcopenia, which encompasses both of these loses has been associated with numerous adverse health outcomes (See section 1.5.).

1.5. Body composition changes with age

A decline in muscle mass is not the only change in body composition that occurs with ageing. An increase in percentage fat mass has been recorded by a number of longitudinal studies as outlined by (Kyle *et al.*, 2001). Furthermore, it is not just the overall increase in

percentage body fat, but the distribution of body fat amongst different areas of the body (Visser *et al.*, 2005; Ponti *et al.*, 2020). The differences in fat distribution led to the identification of an increase in fat infiltration within the muscle with age (Borkan *et al.*, 1983). Resistance exercise training has been shown to alleviate this fat infiltration. For example, a study using 12-weeks of lower-extremity resistance exercise training in 32 older individuals found a significant decrease in thigh intermuscular adipose tissue (IMAT) (Marcus *et al.*, 2010).

Bone mineral density (BMD) which can be measured by DXA also declines with age, which can result in an increased risk of fractures (Marshall, Johnell and Wedel, 1996).

1.5.1. Alterations in muscle protein turnover with age

As the muscle mass is controlled by a dynamic equilibrium between MPS and MPB, where when the rate of MPS equates the rate of MPB muscle mass is maintained. Investigating protein turnover is important to understand what may be driving the change in muscle mass, particularly in age where a decline in muscle mass is evident. Basal protein turnover has been reported to be similar between both young and old individuals in studies where muscle synthesis and proteolysis was measured simultaneously via AV balance (Volpi *et al.*, 2001; Paddon-Jones *et al.*, 2004). Additionally, no significant differences are found in postabsorptive FSR (fractional synthetic rate; See Section) between young and old (Paddon-Jones *et al.*, 2004;

Katsanos *et al.*, 2006; Mitchell *et al.*, 2015). However, differences lie in the response to anabolic stimulus between young and old. Numerous studies have shown a diminished FSR increase in elderly post EAA feeding, suggesting that older individuals require a greater amount of protein to elicit the same MPS response (Cuthbertson *et al.*, 2005; Moore *et al.*, 2015). These studies highlighted the "anabolic resistance" or blunting in older individuals.

The methods used to measure muscle protein synthesis and muscle protein breakdown will be described in more detail in sections 1.6 and 1.7.

1.6. Sarcopenia

1.6.1. Sarcopenia: brief overview

Initially, sarcopenia was simply defined as the loss of muscle mass with advancing age (Rosenberg, 1989), as taken from the direct Greek translation of "sarx"- flesh and "penia"- loss (Rosenberg, 1997). However, since then *dynapenia* i.e. the loss of muscle strength and function with age, has integrated itself within the original definition (Rosenberg, 1997; Mitchell *et al.*, 2012). In 2016, sarcopenia was identified as a disease and awarded the M62.84 code in the ICD-10-CM (Anker, Morley and von Haehling, 2016; Cao and Morley, 2016). This should aid health professionals in identifying sarcopenia within the clinical population. However, since 1989 numerous definitions and ways to assess sarcopenia have been used and with the slight variability between them, results in the assessment of sarcopenia may not be comparable or consistent. The

effect of this on the epidemiology of sarcopenia has been outlined in a meta-analysis by Mayhew *et al.*, 2019 . As the UK is experiencing an ageing population, identifying individuals who are at-risk of sarcopenia or sarcopenic is crucial in order to be able to intervene and aim to minimise that loss.

1.6.2.The Evolution of Definitions of Sarcopenia

In 2009 the International Working Group on Sarcopenia (IWGS) defined sarcopenia as "the age-associated loss of skeletal muscle mass and function" (International Working Group on Sarcopenia, 2011). The IWGS suggested that all older patients presenting declines in physical function, strength or even overall health should be considered for sarcopenia assessment. Individuals would be deemed sarcopenic if they cannot independently rise from a chair or have a measured gait speed >1m/s. The following year, the European Working Group on Sarcopenia in Older People (EWGSOP) published their own considerations for the diagnosis of Sarcopenia. They considered sarcopenia as a "geriatric syndrome" due to the additional factors contributing to the loss of muscle mass and function other than age such as, sedentary lifestyle, chronic diseases and medications (Cruz-Jentoft *et al.*, 2010). The flowchart the EWGSOP suggested for the diagnosis of sarcopenia is presented in Figure 1.5.1. The group further proposed to categorise the diagnosis as 'primary' if age was the only factor resulting in sarcopenia or 'secondary' if there were other contributing factors. Additionally, a grading structure was designed to assess the severity of sarcopenia (See **Table 1.6-1**).

	Criteria 1	Criteria 2	Criteria 3
	Low muscle mass	Low muscle strength	Decreased physical function
Presarcopenia	✓		
Sarcopenia	✓	✓	
Severe sarcopenia	✓	✓	✓

Table 1.6-1 Grading structure for the severity of Sarcopenia suggested by the EWGSOP

In 2014 the Foundation for the National Institutes of Health (FNIH) published their recommendations for sarcopenia. Their clinical paradigm suggested to test for sarcopenia once a patient presents with poor physical function i.e. a gait speed less than 0.8m/s (Studenski *et al.*, 2014). Once this is presented, muscle strength is to be measured and only if low to then assess muscle mass to diagnose for sarcopenia. If strength and mass are above the cut-off points, then sarcopenia is not the cause for poor physical function (See Figure 1.6.2.1).

In 2018, the EWGSOP released an updated version of their 2010 recommendations (Cruz-Jentoft *et al.*, 2018). The main changes in the algorithm for screening include the suggested use of the SARC-F questionnaire followed by initial evaluation of muscle strength before pursuing with further assessments of sarcopenia. SARC-F is a simple questionnaire, consisting of 5 questions that assess strength, walking ability, ability to rise from a chair, climb stairs and frequency of falls (Malmstrom and Morley, 2013). However, the alternative to the use

of SARC-F at screening is ‘clinical suspicion’ which can be deemed subjective. Therefore, in order to reduce the variance at the initial screening process, there should be a unanimous decision across all scientific bodies whether to fully implement SARC-F or not.

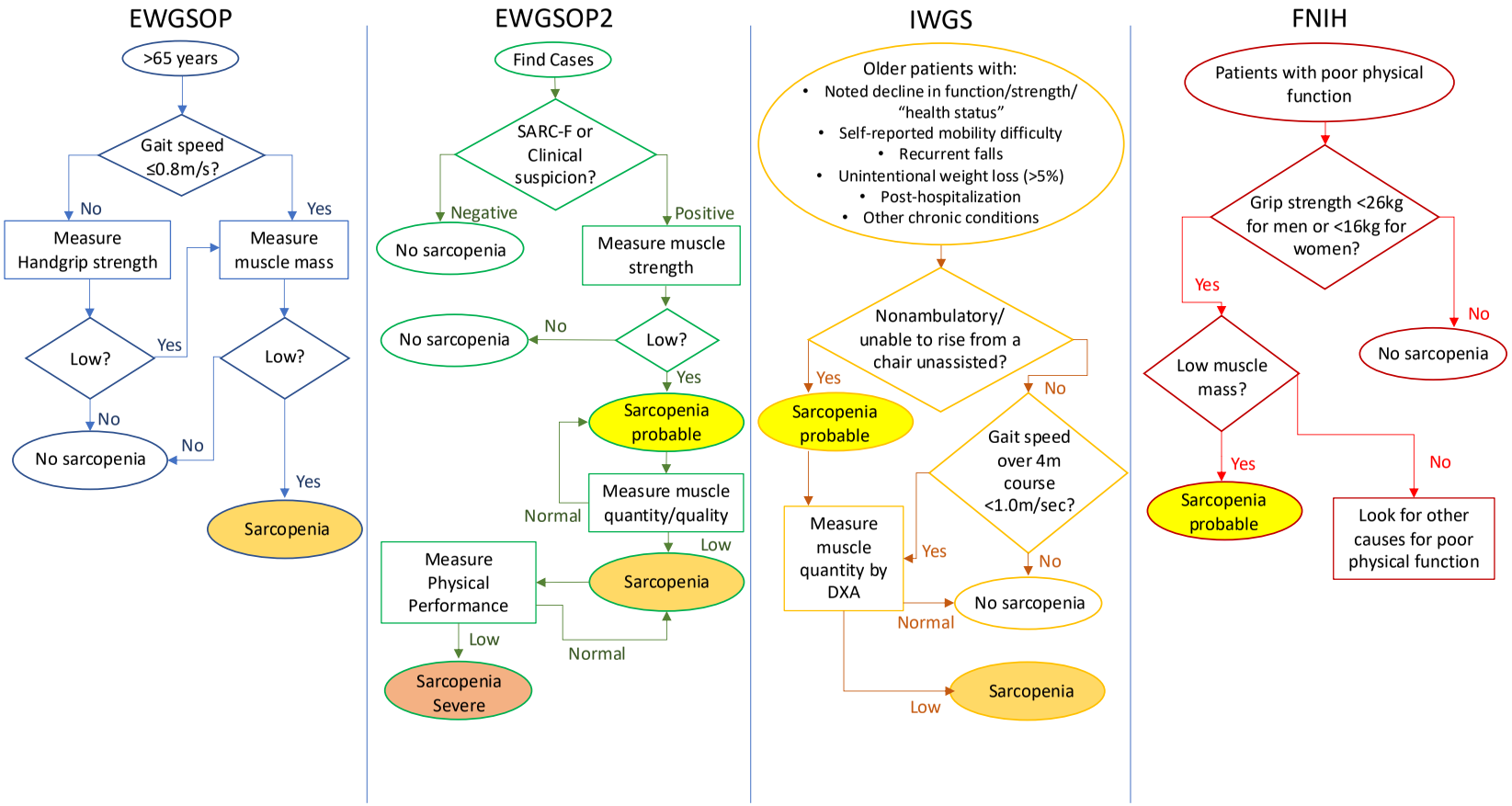


Figure 1.6.2.1 A comparison of the definitions of sarcopenia developed by the European Working Group on Sarcopenia in Older People (EWGSOP), including their updated version (EWGSOP2) and by the International Working Group on Sarcopenia (IWGS) and the Foundation for the National Institutes of Health (FNIH).

1.6.2.1. *Assessment of Muscle Mass in Sarcopenia*

In addition to the differences in definitions and paradigms to identifying sarcopenia, discrepancies lie between the definitions in the methods and cut-off values used to diagnose sarcopenia. MRI/CT scans are considered the most accurate methods in measuring lean muscle mass and therefore, are more routinely used in research; however, these techniques are expensive and largely inaccessible for a clinical population. It was therefore, agreed that DXA or BIA measurements can be used for the assessment of sarcopenia (Cruz-Jentoft *et al.*, 2010). Using these measures, an equation was formulated and defined as the Skeletal Muscle Index (SMI), which is calculated as: (skeletal muscle mass/ body mass) x 100 (Janssen, Heymsfield and Ross, 2002). If using DXA, this equation differs slightly as DXA does not provide an accurate measure of skeletal muscle mass; therefore, the SMI using DXA is calculated using the appendicular lean mass (ALM) /height^2 (Cruz-Jentoft *et al.*, 2010). Individuals with a SMI that is between -1 to -2 standard deviations (SDs) from the sex-specific mean of the young (18- 39 y) adult values; are considered to be class I sarcopenic (Janssen, Heymsfield and Ross, 2002). Class II sarcopenia is characterised as individuals who are found to have an SMI less than 2 SDs of the mean of the young adult reference values (Janssen, Heymsfield and Ross, 2002). Janssen et al. (2002) had used the National Health and Nutrition Examination Survey III for the young adult reference values, as for their study they had measured skeletal muscle mass using BIA. A majority of research studies that have used DXA have used the

Rosetta study (D Gallagher *et al.*, 1997) to obtain young reference values (Fielding *et al.*, 2011).

1.6.2.2. *Assessment of muscle strength and function in Sarcopenia*

Handgrip strength is regularly used as a marker for muscle strength in elderly (Giampaoli *et al.*, 1999; Taekema *et al.*, 2010) and is used in the assessment of sarcopenia (Cruz-Jentoft *et al.*, 2010). The updated EWGSOP definition of sarcopenia has updated the handgrip strength cut-off values for both males and females. The impact of this change is to be investigated.

The Short Physical Performance Battery Test (SPPBT) is a well-established group of exercises used to determine physical frailty (Guralnik *et al.*, 1994). The SPPBT is also included by the EWGSOP in the assessment of sarcopenia. The SPPBT consists of three separate tests assessing balance, gait speed and unassisted chair rises. The maximum score is 12 if all tests are completed effectively. The EWGSOP states that a score of 8 or less determines an individual as sarcopenic based on muscle function.

Gait speed, although part of the SPPBT, can be used as a determinant of muscle function. The EWGSOP state that a walking speed of <0.8m/s distinguishes sarcopenic individuals (Cruz-Jentoft *et al.*, 2010). In contrary, the IWGS use a walking speed of <1m/s as the cut-off for sarcopenia (International Working Group on Sarcopenia, 2011).

1.6.3.Countermeasures to Sarcopenia

To aid the ICD-CM-10 code for sarcopenia, a list of suggested treatments has been made for clinicians. Sarcopenia may be treated with resistance exercise (Peterson and Gordon, 2011), leucine supplementation, alongside vitamin D supplementation (Anker, Morley and von Haehling, 2016). Testosterone provision has also been suggested (Anker, Morley and von Haehling, 2016; Cao and Morley, 2016) as it is known to increase muscle mass and function (Bhasin *et al.*, 1996). However, muscle mass has been shown to be frequently responsive to resistance exercise in the older population (Churchward-Venne *et al.*, 2015).

1.6.3.1. *Resistance exercise in older adults*

In comparison with endurance exercise (also known as aerobic exercise), resistance exercise (RE) can be identified as a set of high intensity contractions against an external force performed over a shorter period of time, such as weight training (Kumar *et al.*, 2012). Progressive resistance exercise significantly increases muscle mass (Hunter, Mccarthy and Bamman, 2004; Phillips *et al.*, 2012) and improves muscle strength (Fielding, 1995; Liu and Latham, 2009). The majority of RE training programmes involve 2-3 sessions of strength training/week and current evidence is based on training programmes lasting up to 12 weeks which supports current UK physical activity guidelines for older adults (>65 years) of at least 2 days/ week of muscle strength building exercises such as lifting weights or chair aerobics (Davies *et al.*, 2019). However, adherence to such strength training programmes can be difficult to achieve due

to a number of barriers, whether socio-economic, environmental or psychological (Zlot *et al.*, 2006). As such interventions such as home-based resistance exercise training that may overcome such barriers have been investigated and are being developed (Thiebaud, Funk and Abe, 2014; Luger *et al.*, 2016).

1.7. Methods for Assessment of Muscle Mass

There are numerous different techniques (anthropometric, imagery or urinary) that can be used to assess skeletal muscle mass. The following section will provide a brief introduction to the main imagining techniques in current use and the proposed tracer technique.

1.7.1. Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) is a non-invasive imaging technique that has long been recognised as a reliable measure for body composition and gold-standard in the estimation of skeletal muscle mass. Since the development of this technique in 1974 by Sir Peter Mansfield and Paul Lauterber (Geva, 2006), this method is capable of producing high resolution images that can detect changes in muscle volume, fat infiltration and also inflammation (Meyer and Prior, 1999). The instrumentation was developed from the nuclear magnetic resonance (NMR) technique (Geva, 2006). The MRI can provide measurements from the realignment of atomic nuclei using an external magnetic field, then switching the field off to observe the "relaxation" period of nuclei, during which the composition of the area under observation can be determined (Lukaski, 1998). The accuracy of this method is increased by using multiple slice images (Thomas *et*

al., 1998). Whole-body skeletal muscle mass can be estimated through numerous sequences of axial slice images, which tend to be set at a 10 mm thickness at 40 mm intervals (Ross *et al.*, 1996; Hellmanns, McBean and Thoirs, 2015). The error in reproducibility of MRI for adipose tissue is <3% (Lustgarten and Fielding, 2011). Furthermore, MRI has been able to detect age-related changes in muscle size and muscle fibre composition (Houmard, Smith and Jendrasiak, 1995). Madden-Wilkinson et al. (2013) found that MRI identified a greater decrease in thigh muscle size with age in males (27%) and females (21%), than DXA (males: 21%, females: 11%). This method does not involve any exposure to radiation which makes it safe and an extremely suitable measure for a wider population, such as infants or pregnant women. Despite the safety and accuracy of this technique's estimation of skeletal muscle mass; the applicability of use in the wider clinical population is limited. This comes as a consequence of limited instrument availability, the expense of the technique as well as, limited access to accurate analysis of images (Wang *et al.*, 2003).

1.7.2.Computed-Tomography

Both computed-tomography (CT) and MRI scans have been validated and recognised as accurate techniques in the assessment of skeletal muscle mass (r= 0.99) (Mitsiopoulos *et al.*, 1998). The CT technique is in constant close competition with MRI for the accuracy of measure of body composition and similarly was developed in the 1970s (Geva, 2006). However, unlike MRI, this method relies on the use of x-rays

to observe the reduction or attenuation, in the intensity of the image, as they pass through different tissues of the body (Lukaski, 1998). Similar to MRI, the CT scan can either use a multi-slice imaging technique for whole-body skeletal muscle measurements or a single abdominal cross-sectional image (Shen *et al.*, 2004). CT has been able to detect changes in muscle mass and muscle quality with age. Using CT, Borkan et al. (1983) identified fat infiltration into the muscle that occurs with age. Unfortunately, this technique involves a high dose of radiation which makes it an unsuitable technique to measure skeletal muscle mass in a larger clinical population (Chen *et al.*, 2007). The radiation dose for a CT scan of the body can range from 2-10 mSv.

1.7.3.Dual-energy X-Ray Absorptiometry

Dual-energy X-ray absorptiometry (DXA) is currently a widely appreciated alternative measurement for assessment of body composition i.e. lean tissue mass, fat mass and bone mineral content (Chen *et al.*, 2007; Cruz-Jentoft *et al.*, 2010). The DXA acquires data from a low dose of x-ray beams travelling rectilinearly across the body at two different energy levels (Visser *et al.*, 1999; Guglielmi *et al.*, 2016). The radiation dose from DXA is extremely low with approximately 0.001 mSv which is the equivalent to less than a day with natural background radiation. The attenuation of the radiation will differ depending on the energy level and body tissue composition (Guglielmi *et al.*, 2016). Tissues of a higher density will attenuate more of the x-ray beam than lower density tissues (Guglielmi *et al.*,

2016). It is a relatively accurate form of measuring skeletal muscle mass, that is cheaper, more widely available and less time-consuming than MRI (Hellmanns, McBean and Thoirs, 2015). Chen et al. 2007 showed a strong correlation between DXA appendicular lean muscle mass and MRI measured muscle mass (r= 0.95). Despite, this positive correlation, known a disadvantage to the use of DXA is its' overestimation of skeletal muscle due to the combined measurement of skin, connective tissue and bone marrow with muscle (Heymsfield *et al.*, 1990). This contribution is minimal in healthy young adults (Heymsfield *et al.*, 1990), however it can become a significant source of error when trying to accurately measure total muscle mass in older individuals (Guglielmi *et al.*, 2016).

1.7.4. Ultrasound

Ultrasound is a method that may be used to assess skeletal muscle thickness and quality (Heymsfield *et al.*, 2014). A transducer probe emits high-frequency sound waves that pass through the skin surface and different body tissues. As they pass they are reflected back to the transducer and an image will be created on the ultrasound screen (Abu-Zidan, Hefny and Corr, 2011). The more dense the material is the more the ultrasound waves will reflect back and produce bright/ white images (Abu-Zidan, Hefny and Corr, 2011). Sanada et al. (2006) through the use of B-mode ultrasonography to measure muscle thickness at nine different anatomical sites, identified prediction equations for total skeletal muscle mass that correlated well with MRI results (Sanada *et al.*, 2006). As ultrasound is a more portable form of measurement of

skeletal muscle mass than DXA or MRI, recent investigations have tried to identify if ultrasound could be used to identify sarcopenic individuals. Minetto et al. (2016) identified cut-off values based on thickness of the rectus femoris, vastus lateralis, tibialis anterior and medial gastrocnemius in 44 older adults and 60 younger individuals (Minetto *et al.*, 2016). However, more studies are required to identify cut-off values for a range of ethnic groups and cohorts.

1.8. Methods used for the assessment of Muscle Metabolism

1.8.1. What is a stable isotope?

In 1920, the work of FW Aston and JJ Thomspon with the mass spectrograph (first mass spectrometer) provided the first insight into accurately identifying whole atomic masses, such as Neon (Ne) with two different atomic masses 20 and 22 (^{20}Ne and ^{22}Ne) (Lehmann, 2016; Wilkinson, 2016). Atoms are composed of a core of positively charged protons and uncharged neutrons, circulated by negatively charged electrons. An *isotope* is defined as an atom of an element that only differs in the number of neutrons, whilst the number of protons and electrons remains constant, as expected with the element (Wilkinson, 2016). Consequently, the isotope will only differ in mass whilst the chemical nature of the atom will remain unchanged (Wolfe and Chinkes, 2005). These properties of isotopes provides the feasibility of their use as tracers in metabolic studies as they can act "identically" to the endogenous atoms but, due to their different mass

and the development of mass spectrometers, they can be traced (Wilkinson, 2016). The numbers stated before the element define the atomic mass of the element. Additionally, isotopes can be referred to as *stable* or *radioactive.* Although not always the case, the most abundant isotope of an element is the one with the lowest mass; for example, ^{1}H is the most abundant atom, ^{2}H (deuterium) is the stable isotope (0.015% abundance) (Garlick, McNurlan and Caso, 1997) and ^{3}H (tritium) is the radioactive isotope of the element.

Between 1950 and 1970 the use of radioactive isotope tracers in metabolic studies was highly popular due to their availability, as a result of the nuclear arms race, and ease of measure (Wolfe and Chinkes, 2005). However, the ionising nature of radioactive isotopes is potentially unsafe as the number of additional neutrons in the nucleus of the atom causes too great of an imbalance between the protons and electrons and the atom may begin to spontaneously decay, generating ionising radiation that can damage cells. Despite the apparent popularity of radioactive isotopes in metabolic research, stable isotopes were in fact the first to be used in metabolic research as early as the 1930s by Harold Urey and Rudolf Schoenheimer (Wilkinson *et al.*, 2015). Unlike radioactive isotopes, stable isotopes do not spontaneously decay which makes them safer and more widely applicable in human metabolic studies (Busch *et al.*, 2006). In metabolic studies it is possible to incorporate isotopes (either radioactive or stable) into any compound (the tracer) that will presumably be chemically identical to the endogenous compound (the *tracee*) (Wolfe and Chinkes, 2005). The difference in isotopic mass

permits the abundances of the tracer and tracee to be distinguished and measured by mass spectrometry.

1.8.2.General introduction to mass spectrometry

As mentioned previously, the development of mass spectrometers has provided the capability of accurately measuring atomic masses. To understand how it is possible to measure muscle metabolism through isotope tracer work, it is important to understand how the read outs are obtained from the samples on the mass spectrometer. The diagram below depicts a brief representation of the components of a mass spectrometer and how they are interlinked.

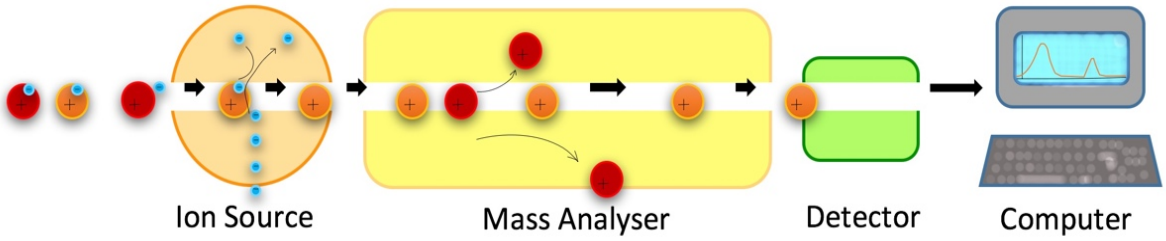


Figure 1.8.2.1 Basic depiction of sample analysis by mass spectrometry

The basic principle behind the distinction of different isotopes by mass spectrometers is their ability to detect different mass: charge (m/z) ratios. An ion source is used to convert the sample gas into positively charged ions by energetic electrons passing by the atom and displacing its electron (Mallet and Down, 2009). This process is termed ionisation and can be represented by the following equation:

$$\mathbf{M + e^- \rightarrow M^+ + 2e^-}$$

(Hoffmann, 2005)

Once the ions have been generated they are separated accordingly through a mass analyser under vacuum before the m/z's are identified by the detector (Hoffmann, 2005). Depending on the mass analyser used, once the ions have been formed they will travel through electrical or magnetic fields and separated by their m/z (Mallet and Down, 2009), to be detected and finally provide a read out on the computer in the form of a chromatogram. Despite a wealth of differing mass-spectrometers available, all of which vary in sensitivity and specificity, the work presented in this thesis involved three different types of mass spectrometers: i) Gas Chromatography-Pyrolysis-Isotope Ratio Mass Spectrometry, ii) Liquid Chromatography Mass Spectrometry and a iii) High-Thermal Conversion Elemental Analyser.

1.8.3.Gas Chromatography-*Pyrolysis*-Isotope Ratio Mass Spectrometry

An isotope ratio mass spectrometer (IRMS) has the ability to provide a continuous and highly precise measure of two or three isotope mass ratios i.e. (m+1)/m at the same time (Meier-Augenstein, 1999). For compound separation, a gas chromatographer (GC) can be attached to the IRMS. However, for the IRMS to be able to detect isotopes of hydrogen/ carbon/ nitrogen or oxygen, the product needs to be in the form of a simple gas. Therefore, a combustion or pyrolysis (incomplete combustion) interface is added in order to convert the analyte separated by GC into a fixed gas such as H_2/CO_2/N_2 or O_2. As H_2 and CO are by-products of incomplete combustion, the GC-

pyrolysis-IRMS system can be used to measure the isotopes ^{1}H and ^{2}H, (Sessions *et al.*, 1999). As amino acids are very polar compounds, they need to be made less polar and more volatile to aid separation. For this to be achieved the sample needs to undergo a process termed 'derivatisation' (Halket *et al.*, 2005; Płotka-Wasylka *et al.*, 2015). Post-injection, once the sample has been separated by the GC it is then eluted into a reactor chamber where it will undergo a pyrolysis reaction. The optimal temperature for the reactor is ~1400°C (Sessions, 2006). Faraday collector cups then act as a detector as the ions that strike the wall of the metal cup and produce a current (Prokůpek *et al.*, 2014). The intensity of the different ions is then measured (Sessions, Burgoyne and Hayes, 2001).

1.8.4.Liquid Chromatography Mass Spectrometry

As previously mentioned, coupling of chromatography with mass spectrometry, allows separation of a metabolite before detection (Lu *et al.*, 2008). This can either be in the form of gas or liquid chromatography (Lu *et al.*, 2008). In contrast to gas chromatography, liquid chromatography requires less sample preparation as the sample does not need to be derivatised to improve volatility (Wilkinson, 2016). Liquid chromatography mass spectrometry (LC/MS) offers extremely high sensitivity (Yates, Ruse and Nakorchevsky, 2009). It is able to measure a range of volatile/ non-volatile, organic or inorganic compounds that the GC-MS is unable to (Vestal, 1984).

1.8.5.TC- EA- IRMS

There are 2 types of elemental analysers (EAs) that can be used to convert a liquid or a bulk solid sample into fixed gases such as CO_2, H_2 and N_2 (Zhang *et al.*, 2012). High temperature thermal conversion elemental analysers coupled to an IRMS (TC-EA-IRMS) can be used (Carter and Barwick, 2011) to measure hydrogen or oxygen isotopes. The system works at high temperatures between 1350-1450°C in the reactor. As the TC-EA is connected to an IRMS for isotopic analysis, faraday cup collectors will detect the ions. For the detection of hydrogen ions, the intensities of m/z's of 2 and 3 ($^1H^1H$ and $^1H^2H$, respectively) are recorded (Carter and Barwick, 2011).

1.9. Intravenous infusion and stable isotope approaches to measure muscle protein synthesis and muscle protein breakdown

1.9.1. Intravenous infusion of stable isotope labelled tracer; the primed constant infusion method

Essential amino acids are often used as tracers as they are not synthesised within the human body (Waterlow, 1995). Non-essential amino acids would affect the relative isotope abundance of the tracer due to *de novo* synthesis which would result in dilution of the isotopic tracer unpredictably (Beynon and Pratt, 2005) in measurements of whole-body kinetics. The introduction of labelled essential amino acid

tracers into the body can be done using one of two methods; primed constant infusion or the flooding dose method.

1.9.1.1. *Primed constant infusion*

The use of primed constant or continuous infusion is one of the most common tracer methods used to measure muscle protein turnover rates (Rennie, 1999; Burd *et al.*, 2011). This is a tracer delivery method that involves intravenous infusion of a stable isotope (or previously used radioactive) labelled essential amino acid, such as phenylalanine. The infusion should reach a steady state of labelling so that incorporation of the tracer into muscle can be measured (Rennie, 1999; Wolfe and Chinkes, 2005). The priming of the tracer into the body involves an initial infusion dose at a slightly quicker rate (i.e. a "priming" dose), in order to reduce the time it takes to achieve a plateau or "pseudoplateau" state of enrichment (Waterlow, 1995; Garlick, McNurlan and Caso, 1997). However, it is also possible for this method to be done without the prime (Rennie, Smith and Watt, 1994), although this would take longer. This tracery delivery approach enables the measurement of muscle protein synthetic rates as well as rates of muscle protein breakdown via the AV-balance method (See section 1.9.3.1).

The principle behind the use of this product-precursor method was to deliver the labelled tracer in an approach that would achieve a steady-state or plateau level of enrichment within the precursor pools for MPS (Garlick *et al.*, 1994). When a plateau state of enrichment has been achieved, the rate of tracer appearance equals the rate of tracer disappearance (Waterlow, 1995). Using the primed constant infusion

the tracer aims to label 5-10% of the free amino acid pool (Rennie, Smith and Watt, 1994). Ideally, the precursor labelling of the aminoacyl transfer RNA (tRNA) would be used as this is the true precursor pool. However, the concentrations of the tRNA pool within a tissue are extremely small and can be difficult to isolate (Watt *et al.*, 1991; Garlick, McNurlan and Caso, 1997). Therefore, it is possible to use either the intramuscular or plasma free amino acid pool as a surrogate precursor (Rennie, 1985). Nonetheless the use of the intramuscular amino acid pool is considered to provide a greater representation of the tRNA pool than plasma (Smith, Patterson and Mittendorfer, 2010).

The disadvantage to this method is that it is quite invasive with the use of an intravenous infusion pump and subsequent blood sampling. Further, it requires the participant to be bed-bound in a controlled clinical setting. This method cannot be used in studies measuring protein turnover rates over 12 hours continuously as the recycling of labelled amino acids can occur which would add ~5% to the steady state enrichment (Waterlow, 1995). The flooding dose is an alternative method that can be used for tracer administration with the aim of improving labelling gradients in the precursor pools (Rooyackers and Nair, 1997).

1.9.2. Assessment of muscle protein synthesis using intravenous tracer infusion methods.

1.9.2.1. *The arteriovenous balance method to measure MPS*

The arteriovenous (AV) balance method can be used to measure MPS and MPB rates. Through the infusion of an isotopically labelled AA tracer, followed by subsequent blood and/or muscle biopsy sampling, amino acid kinetics across a limb or organ can be measured. This method also requires an estimate of blood flow via ultrasound or CEUS. This can be via a 3-pool or 2-pool compartmental model (See Figure 1.9.2.1). The rate of disappearance from the arterial and/or intramuscular pool is representative of muscle protein synthesis and the rate of appearance in the venous pool is demonstrative of muscle protein breakdown (Wilkinson *et al.*, 2015). Volpi *et al.* (2001) have demonstrated that there is no significant difference between the use of the 2-pool or 3-pool model in the basal rates obtained from the amino acid kinetics in young or old subjects. The use of this method in the calculation of muscle protein breakdown will be discussed in section 1.9.3.1.

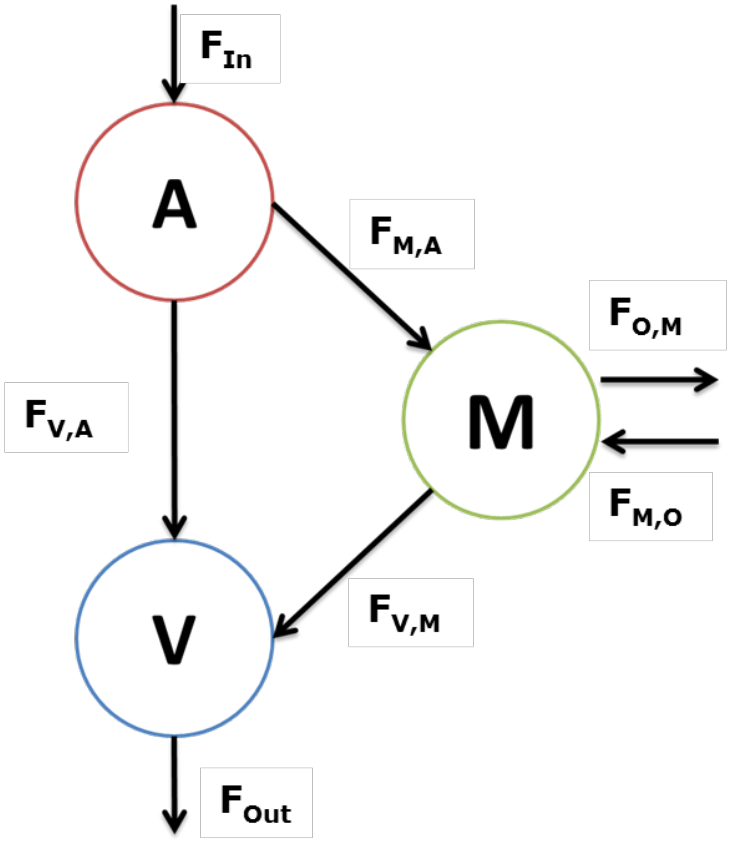


Figure 1.9.2.1 A representation of the 3-pool arteriovenous balance method where A represents the arterial pool, V the venous amino acid pool and M the intramuscular amino acid pool. The direction of the arrows is representative of the flow of the amino acid.

1.9.2.2. *Direct incorporation-precursor product approach to measure muscle protein synthesis.*

The calculation of the fractional synthetic rate (FSR) of muscle protein is a proxy for MPS. Following infusion/ingestion of a stable isotopically labelled amino acid tracer such as, 1,2-$^{13}C_2$ -Leucine/$^{13}C_6$ -Phenylalanine, blood/tissue sampling (for precursor labelling) , and a muscle/tissue biopsy (product labelling), it is possible to measure the rate of label incorporated into muscle protein (Yarasheski *et al.*, 1992). This precursor- product calculation can be simplified by the following equation:

$$\textbf{FSR (\% /h)} = \frac{\Delta E_m}{(\Sigma E_p \times t)} \times 100$$

(Mittendorfer *et al.*, 2005; Wilkinson *et al.*, 2015)

In this equation, ΔE_m represents the difference in protein-bound labelled tracer enrichment between two subsequent tissue biopsies, E_p defines the mean precursor enrichment in the pool chosen to represent the precursor (e.g. intramuscular or venous plasma) and t signifies the time between the two biopsies.

Despite the calculation utilising the enrichment difference between two tissue biopsies, on the initial "baseline" biopsy it is possible to substitute the first biopsy for a plasma sample and measure the background labelling in plasma proteins (in tracer niave subjects) and then to measure FSR using a single tissue biopsy (Rennie, 1985; Rennie, Smith and Watt, 1994). This provides a solution into designing a less invasive approach to measuring muscle protein synthesis/breakdown by reducing the number of biopsies. The use of a single muscle biopsy had been validated by Burd *et al.*, 2011 through the use of L-[*ring*- $^{13}C_6$]-Phenylalanine as a tracer and subsequent determination of plasma protein phenylalanine enrichments for baseline values. This approach can only work with the assumption of zero background i.e. the participants have not been previously exposed to the heavy isotope tracers before or have had

enough time for the tracer to leave the system (Burd *et al.*, 2010; Smith *et al.*, 2010).

1.9.3. Approaches to measure muscle protein breakdown using the intravenous infusion technique

1.9.3.1. *AV Balance*

Calculations for synthesis and breakdown rates are dependent on rate of disappearance (R_d) and appearance (R_a) of the endogenous tracee, respectively. The equations below outline the calculations required for the 2-pool AV Balance model.

$$R_a = ((E_A/E_v)-1) \times [A] \times BF \qquad (1)$$

$$NB = ([A]-[V]) \times BF \qquad (2)$$

$$R_d = R_a + NB \qquad (3)$$

Where E_A and E_v represent the tracer enrichment in the artery and the vein, respectively. [A] represents the arterial EAA concentration and [V] the venous which are multiplied by the blood flow (BF) to calculate net balance (NB).

The values obtained should then be corrected to the leg muscle mass of the measured leg. The main advantage of using the 2-pool AV balance model specifically, is the ability of calculating muscle protein breakdown across the tissue without the need for a muscle tissue biopsy.

1.9.3.2. *Fractional Breakdown Rate*

Similar to the principle behind the precursor-product method to measure fractional synthetic rate, fractional breakdown rate (FBR) can be measured by adapting that precursor- product equation (Zhang *et al.*, 1996). This is primarily used to measure fasted FBR. Based on the same principle as with measuring FSR, FBR can be measured using either the constant infusion or flooding dose method, as long as the pools have reached an isotopic plateau state of enrichment. This method measures the rate of dilution of the tracer in the intramuscular pool by amino acids from the arterial pool and from proteolysis of the muscle. Therefore, for this to be measured, once an isotopic steady state has been achieved, the tracer infusion can be stopped, and the arterial and intramuscular enrichment decay curves can be measured for FBR to calculated. The assumption that the tracer is not reincorporated back into the intramuscular free pool with muscle proteolysis, has to be made with this model. (Zhang *et al.*, 1996)

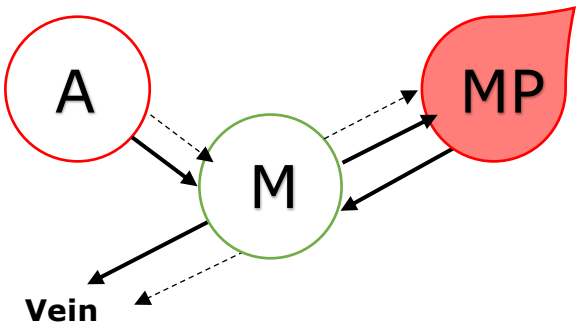


Figure 1.9.3.1 Schematic of movement of labelled (dotted lines) and unlabelled (filled lines) amino acids, from the arterial pool (A) to the intramuscular AA pool (M) as well as to and from the muscle (MP). Adapted from Zhang et al. 1996 & Volpi et al. 2001

depicts the AA pools that are of main concern for the measurement of FBR. It is adapted from the AV balance model, however, unlike the

AV balance model that can function as a two-pool model, the main focus of this method is the intramuscular AA pool (M); therefore, a tissue biopsy would be required for the calculation of FBR. This may be a limitation to the method if a less invasive approach is to be sought. Although, if FSR is going to calculated FBR is a method that can be factored in alongside it.

1.10. Oral stable isotope tracer approaches to measure muscle protein synthesis and muscle protein breakdown

1.10.1. Oral tracer provisions for muscle protein synthesis or breakdown measures

1.10.1.1. *The flooding dose*

The difference between the flooding dose and the primed constant infusion, is the tracer administration approach. Unlike the constant infusion of a labelled tracer to achieve a steady state, the flooding dose involves the administration of a large concentration of labelled and unlabelled amino acids. The aim is to label all the free amino acid pools to the same extent (Smith *et al.*, 1992; Gasier *et al.*, 2009).The advantages to this method are two-fold; firstly, it's easy to measure the precursor pool as the measurement of plasma enrichment imitates the aminoacyl tRNA enrichment and secondly, measurements of MPS can be done over an acute time-frame (approximately 90 min) (Gasier *et al.*, 2009). Despite this, differences

in the FSR values between the two tracer approaches were found. FSR values obtained using the flooding dose were almost 2x greater than FSR values calculated using the primed constant infusion approach (Smith *et al.*, 1992). Additionally, the flooding dose had a greater variability in FSR values than the constant infusion (Smith *et al.*, 1992). The effect of essential amino acids altering metabolism, that was not present with the use of nonessential amino acids as tracers, was also identified through the use of this method (Smith *et al.*, 1998).

1.10.1.2. *Use of D_2O*

Deuterium oxide (D_2O/"heavy water"/ ^2H_2O) was one of the first stable isotope tracers to be used in human metabolic studies (Schoenheimer, Rittenberg and Graff, 1935; Schoenheimer and Rittenberg, 1936; Rittenberg and Schoenheimer, 1937a, 1937b; Foster, Schoenheimer and Rittenberg, 1938; Rittenberg *et al.*, 1938). In 1931, Harold Urey managed to isolate the 'heavy' hydrogen and stable isotope known as deuterium.

In contrast to the previously mentioned stable isotope tracer administration methods, deuterium oxide can be far less invasive as it can be administered orally and rapidly equilibrates with whole body water (Gasier, Fluckey and Previs, 2010; Macdonald *et al.*, 2013). In humans, this tends to be the method of administration, whereas in rats it tends to be provided as an injection. As D_2O incorporates itself with the body water pool, it has the potential of measuring more than one metabolic pathway such as protein (Gasier, Fluckey and Previs,

2010; Wilkinson *et al.*, 2014, 2015), lipid (Bederman *et al.*, 2006; Previs *et al.*, 2011), carbohydrate (Dufner and Previs, 2003; Katanik *et al.*, 2003), DNA (Collins *et al.*, 2003; Busch *et al.*, 2008) or RNA (Defoiche *et al.*, 2009). One of the first studies using deuterium oxide to study protein turnover in rats was in 1941 by Hans Ussing (Ussing, 1941). Here, the transamination reactions involving the transfer of ^{2}H were also observed (Previs *et al.*, 2004). Since then,

the process of deuterium labelling of the α- and β-hydrogens of alanine has been identified (Oshima and Tamiya, 1961). In comparison to the traditional stable isotope methodologies, whereby in order to label the tRNA the labelled stable isotope would have to enter the intramuscular pool via an amino acid transporter, D_2O is able to rapidly label amino acids. D_2O achieves this through by-passing this transporter due to its' incorporation in the body water pool in approximately 2-3h (Gasier, Fluckey and Previs, 2010). As alanine provides approximately four potential hydrogens to be exchanged with deuterium (Dufner *et al.*, 2005), the α- and β-hydrogens of alanine are labelled with ^{2}H approximately 3.7 times more than the hydrogen molecules of D_2O (Gasier, Fluckey and Previs, 2010; Wilkinson *et al.*, 2015). Subsequently, knowing this exchange ratio permits the use of body water via saliva sampling as the precursor pool for protein synthetic rates. This is an additional advantage of D_2O as it is less invasive than the traditional stable isotope approaches that require blood sampling for precursor enrichment measures (Matthew S. Brook *et al.*, 2015; Wilkinson *et al.*, 2015).

As D_2O is stable-isotopically labelled water, it is generally safe for human consumption. However, some reported side effects of D_2O in humans are nausea and vertigo (Jones and Leatherdale, 1991; Gasier, Fluckey and Previs, 2010). These side effects can be minimised or avoided through appropriate dosing (Wilkinson *et al.*, 2015), for instance, by splitting the dose required into smaller doses over time. Deuterium oxide toxicity can occur with extremely large doses of D_2O that result in enrichment levels over 20%. In these cases, side effects can be lethal with cardiac abnormalities, impairment of renal function, differences in reaction rates and hormonal imbalances (Jones and Leatherdale, 1991). In protein turnover studies measuring FSR using D_2O, the dose required for these measures is much lower at approximately 0.5 atom percent excess (APE) which is safe for humans (Katanik *et al.*, 2003; Busch *et al.*, 2006).

An additional advantage to the use of D_2O is its ability to be used in both acute and chronic metabolic studies. Unlike the traditional stable isotope approaches that would require either an infusion or acute measurement of FSR, deuterium has a half-life of ~7-10 days due to the slow turnover rate of body water (Macdonald *et al.*, 2013; Wilkinson *et al.*, 2014). This ensures that FSR can be studied over weeks or months in the "free-living" environment, provided that the enrichment level is maintained (Dufner and Previs, 2003). In 2014 Wilkinson *et al.* identified that through the increased sensitivity and reproducibility of mass spectrometry measures, it was possible to

detect changes in FSR using D_2O over 2-days. Following from that Wilkinson *et al.* applied the use of D_2O against a traditional approach i.e. primed constant infusion of L-[*ring*-$^{13}C_6$] Phenylalanine and found no significant difference between the two methods in measuring the acute (~3h) FSR response to an anabolic EAA feed. This highlighted the use of D_2O in an acute timeframe and demonstrated the less invasive nature of this tracer method in comparison with the primed constant infusion.

1.10.2. Approaches used to measure muscle protein breakdown from oral tracer provision

In addition to the FBR and AV balance method, the third method to investigate muscle protein breakdown in using labelled 3-methylhistidine, which will be described in the following section.

1.10.2.1. *3-Methylhistidine*

In 1954, Tallan, Stein and Moore discovered endogenous 3-methylhistidine (3MH) in human urine, although it's origin was still unclear (Tallan, Stein and Moore, 1954). By 1967, Asatoor and Armstrong confirmed that the source of the endogenous 3MH was, in fact, partially the myosin fragment but, largely the actin fragment of striated muscle (Asatoor and Armstrong, 1967). It was found that 3MH was released upon breakdown of these contractile muscle proteins and it was therefore, suggested that the measurement of 3MH in urine could be used to assess muscle protein catabolism (Kinney, 1975). Through the provision of stable isotope-labelled 3MH as a "tracer" it is possible to measure the dilution of this tracer by

endogenous 3MH released through muscle protein breakdown over time. This can be measured using either urine or plasma sampling (Sheffield-Moore *et al.*, 2014). Young et al. (1972) revealed that once 3MH is released from muscle protein catabolism, it is not reincorporated and used for muscle protein synthesis- a factor of great importance for the functioning of this method. However, conflicting data surrounds this method with regards to the contribution of 3MH from other tissues such as gastrointestinal organs (Long *et al.*, 1988). Different stresses such as trauma, diet and illness have also been studied using 3MH due to the different tissue-specific excretion of 3MH in these conditions (Long *et al.*, 1981; Lowry *et al.*, 1985). However, recently Sheffield-Moore et al. (2014), re-visited the use of this technique and found that the decay curves can be measured over a shorter period of time and that the effect of a meat-free diet was insignificant to the results obtained with a meat diet. They provide promising results for the need for further investigation into this method as a useful non-invasive marker for protein breakdown and for its reintegration into research studies.

1.11. Oral stable isotope tracer approach to measure muscle mass using labelled creatine

To understand how this method works, it is first important to understand creatine metabolism and its biosynthesis within the body.

1.11.1.1. *Creatine Metabolism*

1.11.1.1.1. The importance of Creatine in the body

Creatine (Cr) is an organic compound, more specifically an α-methylguanidine acetic acid, that is naturally found in the body. The greatest abundance of Cr, approximately 98%, is located in muscle (Hunter, 1922). It is a key component in the creatine kinase (CK) reaction which provides highly metabolic tissues (e.g. skeletal muscle, cardiac muscle, brain), a more immediate source of ATP instead of relying on diffusion from the blood (Markus Wyss and Kaddurah-Daouk, 2000). Creatine undergoes a reversible and enzymatic phosphorylation to produce phosphocreatine (PCr) and ADP catalysed by CK. There are four distinctive types of CK isoforms, two of which are tissue specific and two that are mitochondrial specific. CK transfers the γ-phosphate from PCr to ADP resulting in the formation of ATP and Creatine (see Equation 1). The high activity of these CK isoenzymes in skeletal muscles allow an equilibrium of ATP:ADP to exist. This is important for high contracting muscles during exercise.

$$Cr + ATP \rightleftharpoons PCr + ADP + H^+$$

Equation 1. The Creatine Kinase (CK) reaction. Reversible reaction providing an immediate source of ATP in highly metabolic cells.

1.11.1.1.2. Cr Biosynthesis

Cr can be synthesised from a sequence of reactions that begin in the kidneys. The first step involves the amidino group from Arginine (Arg) being transferred to Glycine (Gly). This reaction is catalysed by the enzyme L-arginine:glycine amidinotransferase (AGAT) and yields L-ornithine and guanidinoacetic acid (GAA). GAA is then transported to the liver to undergo the GAMT (S-adenosyl-L-methionine:N-guanindinoacetate methyltransferase) reaction. This methylation reaction yields Cr (See Figure 1.10.2.1).

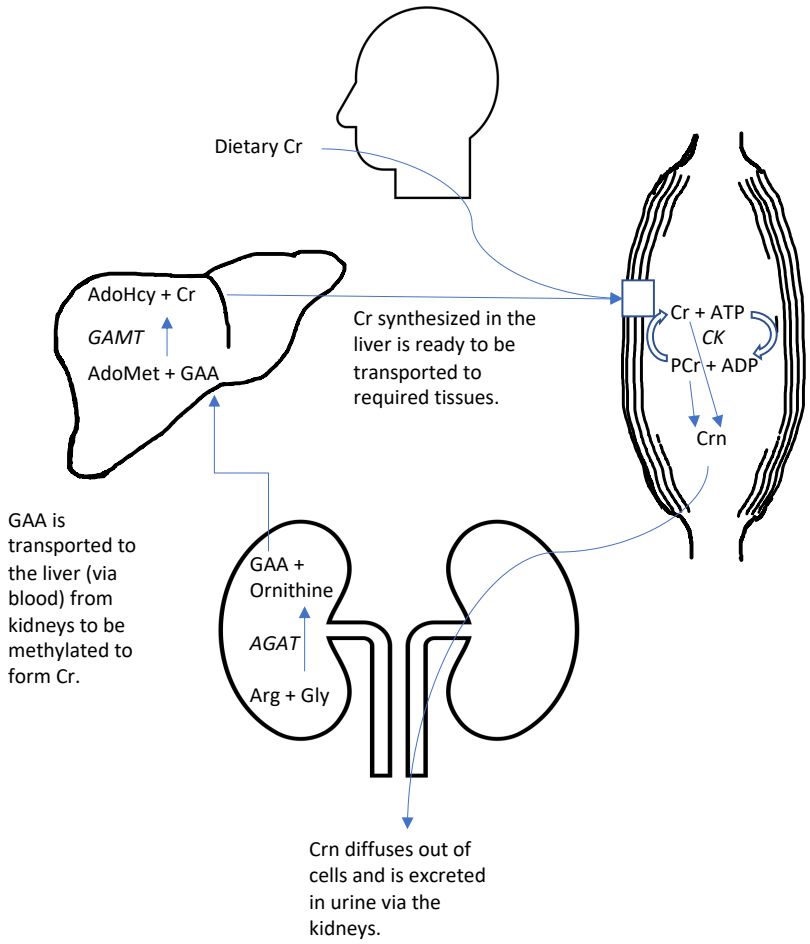


Figure 1.10.2.1 Cr biosynthesis, transport and excretion (Adapted from Wyss & Kaddurah-Daouk, 2000)

1.11.1.2. *Measurement of muscle mass using stable-isotopically labelled creatine*

As mentioned previously, creatine is synthesised in the liver and kidneys before being transported across a large gradient into skeletal muscle. Here it undergoes a reversible phosphorylation into phosphocreatine. Within skeletal muscle creatine is converted irreversibly and non-enzymatically into creatinine, that then diffuses out of the muscle into plasma and is released from the kidneys into urine. (M Wyss and Kaddurah-Daouk, 2000). Approximately 98% of creatine is found in skeletal muscle (Stimpson *et al.*, 2012). Creatine that is ingested orally is also absorbed and transported into muscle (Clark *et al.*, 2014). It is therefore possible to provide a stable-isotope labelled form of creatine (e.g. D_3-Creatine) to trace its equilibration among the creatine pool (primarily within muscle) and observe the conversion into creatinine. Unlike the imaging techniques listed above, the use of this labelled-creatine technique theoretically allows for direct measurement of skeletal muscle mass.

1.12. Summary

Skeletal muscle is a crucial organ involved in the maintenance of whole-body metabolic health. With ageing the loss of skeletal muscle mass and function (i.e. sarcopenia) leads to an increased risk of morbidity and mortality (Cruz-Jentoft and Sayer, 2019). The reasoning for this loss is not yet understood although co-morbidities such as cancer or diabetes cause a more rapid decline in muscle mass. Resistance exercise training has proven to be a beneficial solution in maintaining or at least reducing the progressive rate of muscle

atrophy in older people. In order to assess muscle metabolism and the imbalance in protein turnover, a number of established stable-isotope tracer methodologies have been used to measure rates of MPS and MPB. Additionally, numerous imaging methods have been used for the assessment of muscle mass. However, for the identification of sarcopenia in the wider clinical population (e.g. care homes or smaller community dwellings) the use of expensive imaging techniques is may be impractical. Therefore, it is important to develop less-invasive more accessible methods for the assessment of sarcopenia to gain a greater understanding of the cause of sarcopenia and the potency of nutritional/pharmacological or exercise interventions in mitigating the disease. The use of labelled creatine to measure muscle mass as an alternative to imaging techniques may be beneficial in assessing muscle mass in populations where access to imaging facilities such as DXA or MRI is limited.

The re-emergence and development of D_2O in the measurement of MPS has now been well-established as a less invasive tracer methodology to the more traditional approaches (Wilkinson *et al.*, 2014, 2015). The use of labelled 3-methylhistidine could be a less invasive alternative to the current tracer methodologies used to measure muscle protein breakdown. However, none of these methods have been used simultaneously to measure MPS, MPB and muscle mass in the same older individuals.

As such the central hypothesis of this thesis was that D_2O, D_3-3MH and D_3-creatine could be combined as an approach to measure muscle

protein synthesis, muscle protein breakdown and muscle mass concurrently in older adults.

1.13. Aims and objectives

The aims of this thesis are as follow:

1. Chapter 3. To characterise the older populations who volunteer for research studies, to understand how representative they are of the general population. Furthermore, to compare and contrast the muscle quantity cut-off values and muscle strength and function cut-off values of the four main definitions used for the identification of sarcopenia by the EWGSOP (the original and new version), IWGS and FNIH.
2. Chapter 4. To explore the development and combination of D_3-creatine, D_3-3MH and D_2O methods into one approach to measure muscle mass, muscle protein breakdown and muscle protein synthesis concurrently.
3. Chapter 5. To further assess the combined approach of D_3-creatine, D_3-3MH and D_2O in males and females. To assess any relationships between MPS, MPB, muscle mass and function.
4. Chapter 6. To assess the effects of a 4-week whole-body home-based exercise programme on muscle mass and function whilst utilising the D_3-creatine method for muscle mass.

2. General Methods

2.1. Subject Characteristics and Ethics

Thirty-seven older volunteers (65-85 y; BMI: <35 kg/m^2, 50:50 Male: Female) completed a single 4-day acute study (see Figure 2.2.1.1). Before being enrolled into the study, all subjects underwent a full medical screening. Height, weight, a full medical history, blood pressure assessment, ECG and blood test (Full Blood Count, Urea & Electrolytes, Liver function tests, Thyroid function tests, and coagulation) were used to assess whether the subject is eligible to continue with study.

Subjects were exclude if they had a BMI > 35kg·m^2, any active cardiovascular disease, cerebrovascular disease, respiratory disease, metabolic disease, inflammatory bowel or renal disease, active malignancy, any musculoskeletal or neurological disorders, any signs of clotting dysfunction as well as any recent steroid treatment (within 6 months) or on hormone replacement therapy. All participants were informed of the purpose of the study and of all the risks and procedures involved, before providing their written and informed consent. This study was approved by The University of Nottingham Ethics Committee and conformed to the standards set by The Declaration of Helsinki

2.2. Study Design

2.2.1. Study Protocol

On the first study day (Day 1), volunteers were asked to attend the unit at approx. 8:30 am, fasted, for a whole-body DXA scan. They were asked to provide a saliva sample and then perform a series of simple tests to assess the strength and function in their leg muscles (i.e. short physical performance battery test (SPPBT) and leg extensor/ handgrip strength tests- see 2.3 Baseline Muscle Function Tests) and to complete a quality of life (EQ-5D) questionnaire. A small venous blood sample (~10ml) was taken from the arm before providing the volunteers with the tracer drink containing stable isotope tracers of 30 mg D_3-Creatine in 150ml of D_2O. Following consumption of the tracer drink, participants provided another saliva sample 2 hours later. Volunteers were provided with urine collection tubes, to collect urine for 24h and for a spot sample 48h after the tracer drink. Additionally, they were provided with 10 mg of D_3 -3-methylhistidine (3MH) tracer dissolved in 50mL of ddH_2O to take home and store refrigerated. This was to be consumed immediately after the 48h urine and saliva samples. At 72h post-consumption of the tracer drink (day 4), volunteers returned to site for another study visit, again overnight fasted. This visit involved 6h blood sampling, a urine and saliva sample and a muscle biopsy (under local anaesthetic, 1% lidocaine) from the mid-belly of the vastus lateralis muscle, at 72h. Blood was sampled once every hour for 6 hours (72- 78h), taking ~10ml blood for each sample.

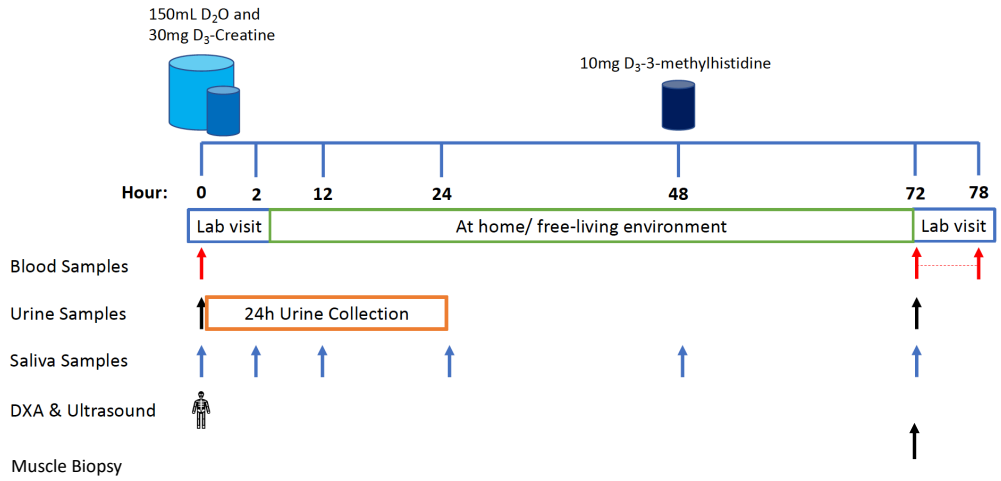


Figure 2.2.1.1 Group 1 Study Protocol

2.3. Baseline Muscle Function Tests

A familiarisation session to all functional tests was not performed.

2.3.1.Short Physical Performance Battery Test

The test consists of 3 exercises that assess the person's balance, gait speed and time-to-stand unassisted. A maximum score of 12 (4 in each test) signifies no frailty. Before each exercise, a demonstration was provided. See Table for an overview of the scoring system.

For the assessment of balance, participants were asked to first attempt a 10 second stand with their feet side-by-side and unsupported. If able to complete, the next balance exercise involved a semi-tandem stand (base of the big toe from one foot, to the heel of the alternate foot), again for 10 seconds. Participants were able to choose which foot they felt more comfortable with placing slightly in front. The final exercise was a full-tandem 10 second stand, heel-to-

toe. If able to hold all stances for 10 seconds, the participant would score full marks.

The second test assesses gait speed. Participants were asked to walk 8-foot from one marked out position to another. They were asked to walk at their normal walking speed, and this was repeated another 2 times. The average speed was taken into the scoring system.

The final assessment of the SPPBT is time-to-stand. A chair with a flat-back and no wheels was placed against a wall to reduce movement. Participants were asked to start from a sitting position, with their arms across their chest. They were to stand unassisted and to repeat these another 4 times whilst being timed from the first stand to the end of the fifth. They were asked to do this as quickly as possible but to stop if it caused them any pain or discomfort.

Points Score	**Assessment**				
	Balance (sec)			**Gait Speed (sec)**	**Time-to-stand (sec)**
	Side-by-side	Semi-tandem	Full-tandem		
0	0-9	0-9	-	Unable to complete	Unable to complete
1	10	0-9	-	>5.7	>16.7
2	10	10	0-2	4.1-5.6	13.7-16.6
3	10	10	3-9	3.2-4.0	11.2-13.6
4	10	10	10	<3.1	<11.1

Table 2-1 An overview of the scoring system used for the SPPBT.

2.3.2.Handgrip Strength

Using the Takei (T.K.K. 5401 GRIP-D) handgrip dynamometer, set so that the middle phalanx would rest on the inner bar, participants would squeeze the bar for 5 seconds to test the strength of the forearm muscles. The test was carried out on each hand with 3 repetitions and at least a 30s rest between each contraction. The protocol followed that of the one used in Great British Boxing where participants were standing, arms by their sides with shoulders adducted at 0° of shoulder and elbow flexion (Gatt *et al.*, 2017). The highest of the three attempts was recorded in kilograms.

2.3.3.Maximal Voluntary Contraction

Maximal voluntary contraction of the dominant leg was assessed using the Cybex Dynamometer. Participants were seated with their dominant leg strapped to the Cybex lever arm. The centre of rotation of the knee joint was aligned to dynamometer fulcrum. The lever arm would then remain fixed at 70° with participants performing 3 repetitions of 5 second leg extensions against the fixed lever. This provided a peak torque measure that was recorded in Newton meters (Nm).

2.3.4.Leg Strength

A single-leg (dominant) extension 1-repetition maximum (1-RM) was used to assess single leg strength using the ISO Lever (Leisure Lines Ltd, GB). After a short warm-up session, participants aimed to reach their maximum within 5 attempts.

2.3.5.Leg Power

Leg power was assessed using the Nottingham Power Rig (Bassey and Short, 1990). Participants were seated with both feet on the foot pedal. Initially, they were asked to push down with one leg so that the seat could be adjusted for leg length, ensuring that when sat back the participants legs could be fully extended without their knees locking at the joint. Arms were crossed over the chest and 5 attempts were recorded. As the legs extend and push down the foot pedal, this turns the flywheel that provides an output measure in Watts.

2.4. Sample Processing and Storage

Upon collection, all muscle biopsy samples were cleared from any excess blood or visible fat and connective tissue, immediately snap-frozen in liquid nitrogen and stored at -80ºC until analysis. Blood samples were centrifuged at (3,200 rpm, 20 min, 4ºC), the plasma aliquoted into eppendorfs and stored at -80ºC. Saliva samples were centrifuged at ~13,000 rpm for 10 min to remove any debris, and then the water was aliquoted into glass vials, capped and frozen at -30ºC until further analysis.

2.5. Muscle Mass Analyses Using D_3-Creatine

2.5.1.Determination of Creatine Concentration and Enrichment in Urine Samples

The following preparation of samples was based on the method by Leonard et al. 2014. Urine samples were freeze-thawed at room

temperature. A standard curve using ^{12}C- and ^{13}C-Creatine was prepared for the determination of creatine concentration (0.25µg to 5µg). A D_3-creatinine enrichment curve of 0-0.1% was also prepared alongside the samples. Once urine samples had thawed and had been vortex mixed, 10 µl of ^{13}C-Creatine was added to 50 µl of urine, as an internal standard. 250 µl of ice-cold acetonitrile was added to each sample and standards, vortex mixed and left to incubate on ice for 30 min. Following incubation, samples were centrifuged at 17,000g for 20 min. The supernatant is then filtered using a 0.2 µm filter and syringe before being transferred to vials ready for analysis on the HPLC/MS. (Leonard, Dunn and Smith, 2014).

From the measurement of urinary D_3- Creatinine enrichment it is possible to calculate total creatine pool size and therefore total muscle through the use of the following equation:

$$\text{Total muscle mass} = \frac{\left(\frac{MW_{Unlabelled}}{MW_{labelled}}\right) \times (Amount\ of\ D_3{-}Cr\ dosed\ (g) - Amount\ of\ D_3{-}Cr\ excreted\ (g))}{(mean\ steady{-}state\ D_3{-}Creatinine\ enrichment\ ratio} \div 4.3(g/kg)$$

(Clark *et al.*, 2014)

Where $MW_{Unlabelled}$ and $MW_{labelled}$ represents the molecular weights of both unlabelled and labelled creatine, respectively. The estimated creatine pool size is then divided by 4.3 g/kg which reflects the concentration of creatine found in whole wet muscle mass.

2.6. Muscle Protein Synthesis Analyses Using D_2O

2.6.1. Measurement of Body Water Enrichment

100 µL of saliva was aliquot into the cap of auto-sampler vials. These were crimped onto inverted vials and placed on a heating block at 90ºC for 4 h. The vials were immediately cooled upright on ice for 10 min and the water collected was transferred to fresh vials. The samples were then ready for injection into a high temperature conversion elemental analyser (TC/EA; Thermo Scientific, Hemel Hempstead, UK) that is connected to an Isotope Ratio Mass Spectrometer (IRMS, Delta V Advantage, Thermo Scientific). (Wilkinson *et al.*, 2015). The mean of 4 repeat injections was taken to control for carry over from previous samples.

2.6.2. Preparation of Muscle for Measurement of Deuterium Enrichment

~30-50 mg of muscle was used for the analysis of deuterium labelling of alanine in the myofibrillar protein bound fraction. The muscle tissue was homogenised with scissors in an Eppendorf tube using 10µl mg^{-1} of ice-cold homogenisation buffer (50 mmol Tris-HCl, 1 mmol EDTA, 1 mmol EGTA, 10 mmol β-Glycerophosphate, 50 mmol NaF, 0.5 mmol of activated sodium orthovanadate, pH 7.4 (all from Sigma Aldrich, Poole, United Kingdom) containing a complete protease inhibitor cocktail tablet (Roche, West Sussex, UK). The homogenate was mixed at 1000 rpm for 10 minutes before being centrifuged at 10,000 x g

for 10 min at 4ºC. The supernatant (sarcoplasmic fraction) was then collected and the pellet was resuspended in 500 µL mitochondrial extraction buffer (MEB) and then homogenized by Dounce and centrifuged at 1000 g for 5 minutes. The myofibrillar pellet was solubilised using 1ml 0.3 $molL^{-1}$ NaOH and incubated for 30 min at 37ºC. The myofibrillar fraction was separated from the insoluble collagen by centrifugation at 10,000 rpm for 10 min. Through the addition of 1ml 1 $molL^{-1}$ Perchloric Acid, the myofibrillar protein fraction was precipitated and incubated at 4ºC for 20 min. After being centrifuged at 4,000 rpm for 20 min, the myofibrillar pellet was washed twice with 70% ethanol. After the addition of 1ml 0.1 $molL^{-1}$ HCl and 1ml of a Dowex H^+ resin slurry, the samples were incubated overnight at 110ºC in order to release the protein-bound amino acids (Philip J Atherton, Etheridge, *et al.*, 2010; Kumar *et al.*, 2012; Wilkinson *et al.*, 2015). The free amino acids were then purified using cation exchange chromatography on Dowex H^+ resin before being eluted with NH_4OH and then dried down. The amino acids (AA) were derivatized to their N-methoxycarbonyl methyl esters (MCME) similar to the protocol previously described by (Hušek, 1998).The AA were re-suspended in 60 µl of distilled H_2O and vortexed mixed before the addition of 32 µl of methanol and 10 µl of pyridine. 8 µl of methylchloroformate (MCF) was carefully added directly into the aqueous mix and immediately vortex mixed for 30 seconds. The solution was left at room temperature for 5 minutes to react. Following the addition of 100 µl of chloroform and 100 µl of 0.001M $NaHCO_3$, the samples were vortexed mixed to extract the MCME

amino acids. Molecular sieve was added to remove any excess water from the sample. The sample was transferred to an autosampler vial, ready for mass spectrometric analysis. Deuterium enrichment into protein-bound alanine was measured using gas chromatography-pyrolysis- isotope ratio mass spectrometry (Delta V Advantage, Thermo Scientific).

2.7. Muscle Protein Breakdown Analyses Using D_3-3-methylhistidine

The following protocol was developed from a previously described method by (Houweling *et al.*, 2012). Plasma samples were defrosted and centrifuged at ~10,000 rpm for 3 min. A 0- 10% D_3-3-methyl-histidine enrichment curve was prepared as a serial dilution. 100 µl of plasma was de-proteinised using 1 ml of MeCN: MeOH (1:1). Samples were vortex mixed and incubated at -20°C for 1 hour. Samples were centrifuged at ~13,000 rpm for 5 min at 4°C. The supernatant was dried down in the Techne Block at <40°C using Nitrogen gas. Samples were re-suspended using 100 µl ACN: ddH_2O (65:35) prior to analysis by High-Performance Liquid Chromatography mass spectrometry (HPLC/MS)

2.8. Muscle Architecture Measurements

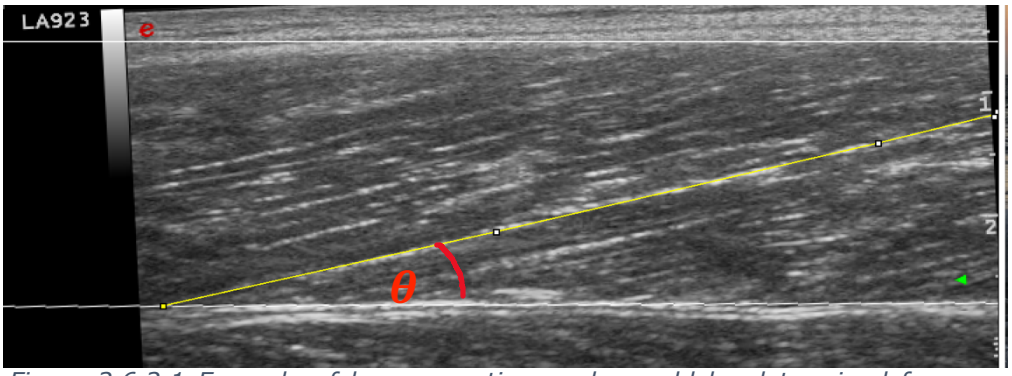


Figure 2.6.2.1 Example of how pennation angle would be determined from a m. vastus lateralis muscle ultrasound image.

On the preliminary visit, each volunteer had the thickness, fascicle length (L_f) and pennation angle (θ_{pen}) of their m. *vastus lateralis* (VL) measured by ultrasound (Mylab 70, Esaote Biomedica, Genova, Italy). Ultrasound measures were obtained whilst the participant was lying supine on a bed for examination. No exercise testing was carried out before imaging and subjects remained supine for ~5 minutes before imaging to allow for body fluid equilibration. All images were obtained by placing the mid-point of the 10cm, 10-15 MHz, linear array probe at 50% of the VL length (i.e. 50% from the greater trochanter to the mid patella) and at 50% of the midsagittal line of the muscle. The probe was then placed along the plane of the fascicles in order to obtain a greater number of fascicles in the image. Ultrasound images were then analysed using ImageJ 1.51g software. Muscle thickness was quantified as the perpendicular distance between the superficial and deep tendon aponeurosis. Pennation angle was measured at the intersection of the fascicle and deep tendon aponeurosis. If the fascicle extended outside the ultrasound

window, linear extrapolation was used to measure fascicle length. (Franchi *et al.*, 2015).

2.9. Dual-energy X-ray Absorptiometry

Lean body mass, fat mass and bone mineral density were all assessed using whole-body dual-energy x-ray absorptiometry (DXA) (Lunar Prodigy, GE Medical Systems, USA) and the software enCORE (version 16, GE Healthcare). Firstly, subjects had an additional safety questionnaire to ensure that they would not be exposed to more ionising radiation than the safe recommendation. Before having the scan, subjects were fasted and asked to ensure their bladder was empty as this could affect the reading. All attenuating materials such as jewellery/belts/metal buttons were removed. The abdominal region was free of metal and no tight waistbands were to be worn during the scan. Subjects were set-up to lie horizontally on the DXA bed, with the centre of their body aligned with the centre line on the DXA bed. The participants head was placed approximately 3 cm below the horizontal line on the DXA bed. The subject's hands were turned on their side, with their palms facing their legs and their thumbs up. The hands were to not touch the legs and a gap of approximately 1 cm was kept between the arms and the torso. The subject was then ready for the total body scan.

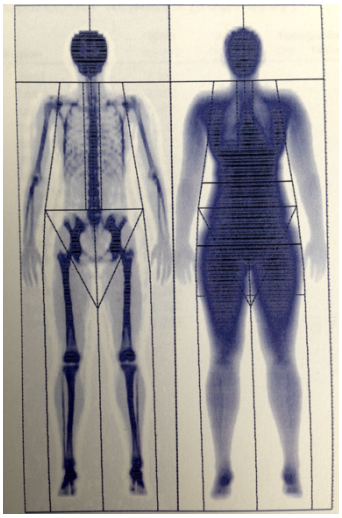


Figure 2.6.2.1 Example of body positioning for DXA

3. Exploring the characteristics of ageing in research participants and the variability in sarcopenia identification

Since the viva voce examination an error in the DXA data, collected by a research colleague, has been identified. It has not been possible to correct the data before submission of corrections. All analyses in the following chapter are based on data that was believed to be correct at the time. Future publications may differ from this Chapter based upon reanalyses of the data.

3.1. Introduction

In order to utilise stable-isotope tracer methods in the assessment of muscle protein turnover and muscle mass in older age, it is vital to understand the population that is being studied and how they may be reflective of the ageing population and range of clinical populations, especially those in which loss of muscle is a feature. Furthermore, for these methods to be used in the assessment of sarcopenia or furthering our understanding of the effects of sarcopenia on muscle protein turnover or vice versa, there needs to be a consensus as to the definition of sarcopenia and methods used to identify it. In brief, sarcopenia is defined as the loss of muscle mass and function with age.

Currently there are a number of different clinical paradigms used for identification of sarcopenia. The most commonly used are those proposed by the European Working Group on Sarcopenia in Older People (EWGSOP) (Cruz-Jentoft *et al.*, 2010), the Foundation of National Institutes of Health (FNIH) (Studenski *et al.*, 2014) and the International Working Group on Sarcopenia (IWGS) (International Working Group on Sarcopenia, 2011). Due to differences observed in ageing based on ethnicity, a consensus was formed by the Asian Working Group on Sarcopenia (AWGS) who follow the same clinical paradigm as the EWGSOP but have conformed their own cut-off values better aligned with the Asian population (Chen *et al.*, 2014). These models differ slightly with respect to the cut-off values that

they use for either the assessment of muscle mass or muscle function, and more recently the EWGSOP have updated their original definition (Cruz-Jentoft *et al.*, 2018), which reduced the percentage of men identified as sarcopenic as was recently highlighted (Reiss *et al.*, 2019). Despite an initial misprint in the updated version by the EWGSOP, whereby the cut-off for appendicular skeletal muscle index (ASMI) was set at 6.0 kg/m^2 for females instead of the now corrected 5.5 kg/m^2, overall these changes resulted in no significant difference in the percentage of women deemed sarcopenic using both definitions remained relatively constant (Reiss *et al.*, 2019).

This study will aim to compare and contrast the muscle quantity cut-off values and muscle strength and function cut-off values of the four main definitions used for the identification of sarcopenia by the EWGSOP (the original and new version), IWGS and FNIH. Furthermore, this study will aim to characterise the older populations who volunteer for research studies, to understand how representative they are of the general population.

3.2. Methods

3.2.1. Subject Characteristics and Protocol

Data from seven research studies previously conducted in our labs, between 2010-2019 including data from the Abbeyfield participants (presented in Chapter 5), that recruited young and elderly, healthy male and female volunteers were used (male: female, 176:100). We compiled their blood results (full blood count, fasting/random glucose,

liver function tests, thyroid function test, coagulation and lipids), muscle mass (body composition) and functional data into a single database to analyse the characteristics/phenotype of these individuals.

Participants were either aged 18 – 35 years or above 65 years and were generally healthy members of the community, who performed regular activities of daily living but were not engaged formally in an exercise programme. Participants were excluded from study if they had a past medical history of diabetes mellitus, cardiovascular disease, pulmonary, thyroid, liver, vascular or kidney disorders or poorly controlled hypertension (>160/100mmHg), were undergoing therapy for cancer or were on a weight-controlled diet. Regular use of NSAIDs, acetaminophen, HRT or corticosteroids and evidence of alcohol excess also excluded the volunteer from being recruited into the studies. The subjects had a normal blood chemistry. All subjects gave their written, informed consent to participate after all procedures and risks were explained. These studies were approved by The University of Nottingham Ethics Committee and complied with the Declaration of Helsinki.

The QRISK®3 (Hippisley-Cox, Coupland and Brindle, 2017) was used to identify the risk of developing cardiovascular disease (CVD) in our volunteers. This algorithm combines the participants age, gender, BMI, systolic blood pressure and cholesterol then calculates a percentage risk of heart attack or stroke in the next 10 years, whilst

also providing an expected readout of expected risk of developing CVD or a stoke in the next 10 years based on age, BMI and gender. We used this to compare our participants risk with the expected risk of the general population at the same age.

3.2.2. Assessment of Body Composition, and Functional Assessment

All volunteers in the database had undergone a Dual-energy X-ray Absorptiometry (DXA) whole body scan. For a detailed overview of this method see section 2.9. From this we analysed bone mineral content (BMC), bone mineral density (BMD), lean body mass (LBM), appendicular lean body mass (ALBM- i.e. total lean body mass of the arms and legs) and % fat mass.

Not all studies had a uniform muscle function assessment protocol. However, where possible we assessed handgrip strength, single-leg 1-RM leg extension, leg power using the Nottingham Power Rig and maximum voluntary contraction (MVC) using a Cybex Dynamometer. Results from a Short Physical Performance Battery Test (SPPBT) were also included, from which the chair test and gait speed were assessed individually. A more detailed explanation of these methods is available in Chapter 2.

3.2.3.Calculation of muscle mass cut-off indices.

The following equations were used to calculate some of the muscle mass indices used to determine sarcopenia.

$$Appendicular\ lean\ body\ mass\ (ALBM) = Total\ fat\ free\ mass\ of\ arms + legs$$

$$Appendicular\ skeletal\ muscle\ index\ (ASMI) = \frac{Appendicular\ lean\ mass\ (kg)}{height\ (m^2)}$$

$$ASMI\ corrected\ for\ BMI = \frac{Appendicular\ lean\ body\ mass\ (kg)}{BMI\ (\frac{kg}{m^2})}$$

3.2.4.Statistical Analyses

All statistical analyses were performed using Prism (version 8, GraphPad Software, San Diego, USA). All data sets were tested for normality using Shapiro-Wilks test. Linear regression and Pearson's Product-moment correlation analysis was used to assess relationships between the variables. Unpaired t-tests were used to assess differences between male and females if data was normally distributed. Mann Whitney U tests were performed for data that was not normally distributed. A two-way ANOVA was used to identify differences between young and old, male and female.

3.3. Results

3.3.1. Subject Characteristics

	Young (18-35y)				Old (65y+)			
	Male		Female		Male		Female	
	Mean	n	Mean	n	Mean	n	Mean	n
Age (y)	24.0 ± 3.5 C,D	57	25.0 ± 3.6 C,D	26	71.5 ± 4.2 D	119	68.2 ± 2.6 A,B,C	74
Height (m)	1.8 ± 0.1 B,C,D	57	1.6 ± 0.3 A,C,D	26	1.74 ± 0.1 A,B,D	119	1.6 ± 0.1 A,B,	74
Weight (kg)	77.5 ± 11.4 B,D	57	68.8 ± 12.6 A,C	26	80.4 ± 10.4 B,D	119	67.1 ± 10.7 A,C	74
BMI (kg/m²)	23.8 ± 3.1 C,D	57	24.9 ± 3.8	26	26.5 ± 2.9 A	119	26.0 ± 3.7 A	74
FFM (kg)	56.2 ± 6.6 B,C,D	57	41.9 ± 5.6 A,C,D	26	53.4 ± 5.3 A,C,D	119	38.4 ± 4.5 A,B,C	74
AFFM (kg)	26.9 ± 5.0 B,C,D	49	18.0 ± 3.5 A,C	21	23.9 ± 4.0 A,B,D	109	16.5 ± 3.0 A,C	70
ASMI (kg/m²)	8.29 ± 1.46 B,D	49	6.56 ± 0.91 A,C	21	7.87 ± 1.09 B,D	109	6.40 ± 1.03 A,C	70
BMD (g/cm²)	1.31 ± 0.1 B,D	57	1.23 ± 0.1 A,D	26	1.28 ± 0.13 D	119	1.07 ± 0.12 A,B,C	68
BMC (g)	3154 ± 397.8 B,C	57	2510 ± 347.9 A,C,D	26	3058± 411.4 B,D	115	2225 ± 398.2 A,B,C	65
% Fat mass	22.6 ± 6.9 B,C,D	57	34.3 ± 6.9 A,C,D	26	29 ± 5.8 A,B,D	119	39.0 ± 5.8 A,B,C	74
QRisk3 (%)	-	-	-	-	19.3 ± 5.9 D	119	10.9 ± 3.0 C	74
Handgrip (kg)	-	-	-	-	37.8 ± 7.7 D	50	28.3 ± 8.0 C	62
1-RM unilateral leg extension (kg)	61.5 ± 16.2 B,C,D	27	37.6 ± 9.1 A,D	18	40.1 ± 11.4 A,,D	30	27.8 ± 9.0 A,C	46

Table 3.3.1-1 Subject Characteristics. Values displayed as mean ± SD. Statistically significant difference to the young male group is represented by A, B- young female group, C-old male group and D-old female group. (Total arms lean mass was missing for some individuals thereby reducing the number ALBM could be measured for).

3.3.2.Blood Chemistry and QRisk3

The full blood count, liver function tests and thyroid function tests were all deemed within range. Based on the estimated glomerular filtration rate (eGFR) for 82 of the older males and 50 older females; 14.6% of the older males and 12% of the older females would be identified as stage 3a chronic kidney disease i.e. an eGFR <60. The QRisk3 identified that the older males had a significantly higher risk of developing cardiovascular disease than the older females in this database. However, both the male and female results were not significantly different to the expected risk for their age/gender/BMI.

3.3.3.Body composition changes with age

There was a significant positive correlation in whole-body % fat mass positively correlated with age in both males and females with age (males: $r=0.455$, $p<0.0001$, $n=176$; females: $r=0.338$, $p=0.0006$, $n=100$). Conversely, BMD decreased with age in both males and females (males: $r=-0.15$, $p=0.044$, $n=176$; females: $r=-0.54$, $p<0.0001$, $n=100$). However, a decrease in BMC only correlated with age in females ($r=-0.36$, $p<0.001$, $n=98$) but not males ($p=0.069$). When focussing on only the older participants >65 years, no significant correlations were observed with age and BMD and BMC. BMD significantly correlated with % fat mass ($r=0.21$, $p=0.024$, $n=119$) in the older male cohort, whereas both BMC and BMD significantly correlated with % fat mass ($r=0.36$, $p=0.0018$ $n=73$; $r=0.39$, $p=0.001$, $n=71$, respectively) in the older females.

3.3.4.Correlation of Bone Indices and measures of strength.

There was a significant correlation between total BMC and BMD with 1RM single-leg extension, as well as BMC and handgrip strength in the older female cohort (See Figure 3.3.4.1)(Outliers had not been removed). However, there were no significant correlations between bone indices and muscle strength (1RM leg extension or handgrip strength) in older men ($p>0.05$).

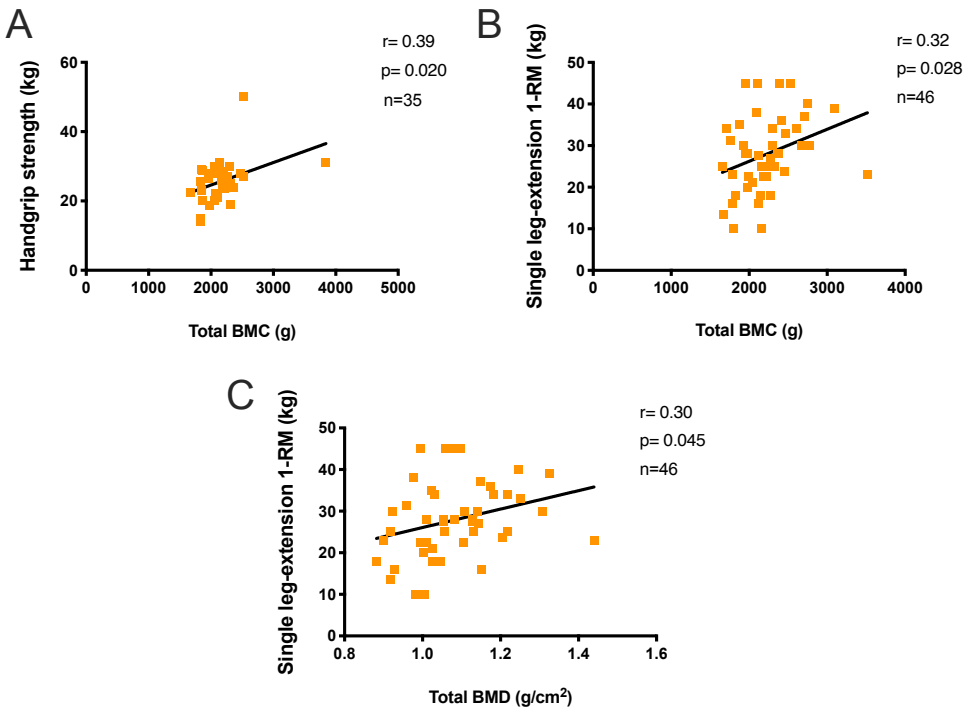


Figure 3.3.4.1 Correlations between (A) total BMC and handgrip strength, (B) total BMC and single-leg extension 1RM and C) single leg-extension 1RM and total BMD, all in the older (>65 years) females.

3.3.5.Effect of Age and Gender on muscle mass

In our cohorts, the older participants had a significantly lower lean muscle mass (as estimated by DXA) than the young (Figure 3.3.5.1). In the older cohort, LBM falls significantly with age in the males but not in the older females (Figure 3.3.5.2).

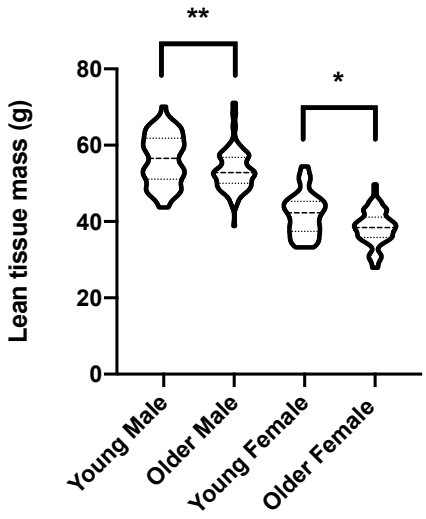


*Figure 3.3.5.1 Young (18-35y) vs. old (>65y) lean muscle mass estimated by DXA and separated male and female. ** denotes p= 0.00991 and * where p=0.023*

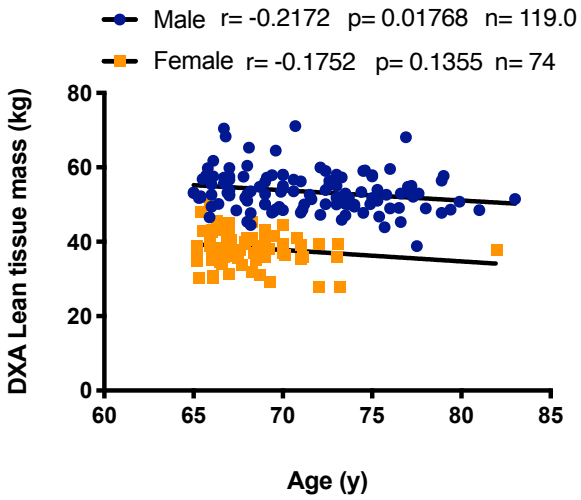


Figure 3.3.5.2 Decline of muscle mass with age within the older cohort (>65y)

3.3.6.Effect Age and Gender on muscle strength and function

Not all participants had performed the same muscle function assessments and consequently there is no data for handgrip strength in the young cohort. There was a significant decline in leg strength

assessed via 1-RM unilateral leg extension with age from young to old (See Figure 3.3.6.1A). When all 1-RM leg extension data for young and old were combined, there was a significant correlation with lean mass estimated by DXA (See Figure 3.3.6.1B).

In the older cohorts (65y+), there was no significant decline in 1-RM strength nor Single leg MVC across age in either males or females, there were however significant negative correlations in both handgrip strength and gait speed and age (Figure 3.3.6.2B and D).

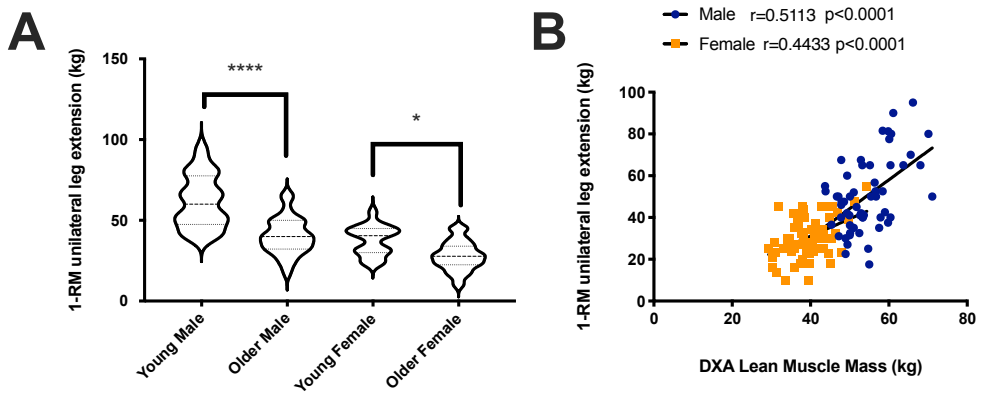


*Figure 3.3.6.1 A) Negative regression of 1-RM unilateral leg extension strength with age. B) A significant positive correlation of muscle mass and 1-RM unilateral leg extension for both males and females. **** denotes p<0.0001 and * where p=0.017.*

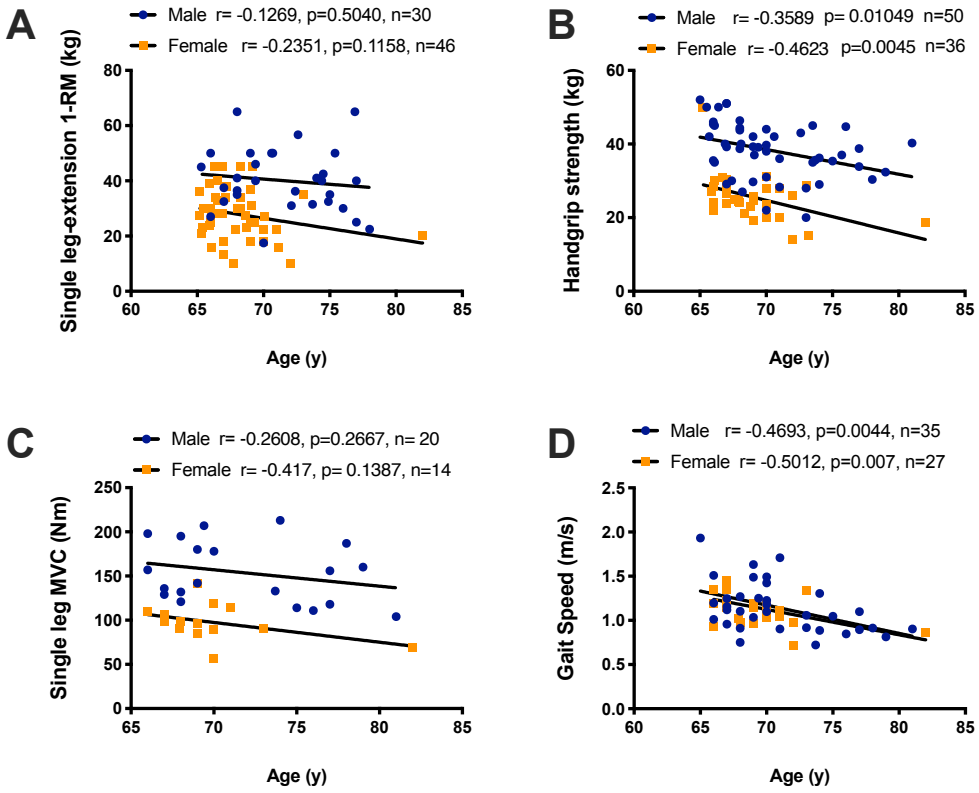


Figure 3.3.6.2 A) Correlations between 1-RM unilateral leg extension B) handgrip strength C) single leg MVC D) gait speed and age in older (65+) males and females.

3.3.6.1. *Application of existing muscle mass cut-offs to define Sarcopenia in our cohorts*

The quantification of ASMI led to vastly different percentages of the cohort being identified as "sarcopenic" based solely on muscle mass, (listed in Table 3.3.6-1) ranging from 6-33% in the older female cohort across the different definitions, and even within the same definition i.e. FNIH utilising appendicular lean mass (ALM) adjusted

for BMI (6%) or ALM alone (33%). Furthermore, the different cut-off values for muscle mass, utilised by the four different definitions for

			Young (18-35y)		Older (>65)	
		Cut-offs	% Male Sarcopenic	% Female Sarcopenic	% Male Sarcopenic	% Female Sarcopenic
FNIH	ALM adjusted for BMI	<0.789 males, <0.512 females	0	0	22	6
	alternate: ALM	<19.75kg males, <15.02kg	2	19	5	33
IWGS	From Health Aging and Body Composition baseline cohort by (Newman et al. 2003) ALM/ht2	<7.26kg/m2 males, <5.67kg/m2 females	31	14	23	20
EWGSOP	ALM/ht2 : From Rosetta study (Baumgartner et al. 1998)	<7.26kg/m2 males, <5.5kg/m2 females	31	10	23	16
	ALM/ht2: Based on sex-specific lowest 20% (Delmonico et al. 2007)	<7.25kg/m2 males, <5.67kg/m2 females	31	14	23	20
	ALM/ht2: Based on sex-specific lowest 20% from Health ABC study (Newman et al. 2003)	<7.23kg/m2 males, <5.67kg/m2 females	29	14	23	20
EWGSOP2	ALM/ht2	<7.0kg/m2 males, <5.5kg/m2 females	20	29	15	16
	ALM	<20kg males, <15kg females	2	19	8	33
		n	49	21	109	70
		SD	14.48	8.29	7.50	8.98

Table 3.3.6-1 Application of different sarcopenia cut-off values for muscle mass provided by the EWGSOP, EWGSOP2, IWGS and FNIH to the young and old participants. ALM- Appendicular lean mass, ALM/ht^2- Appendicular lean mass (kg) divided by height (in m) squared.

sarcopenia, were applied to the younger cohort of our study. The only cut-off that identified 0% of our younger cohort as sarcopenic was the FNIH using ASMI adjusted for BMI. Using the cut-off values of ALM/ht^2 from the original definition of sarcopenia by the EWGSOP, 31% of males from our younger cohort would be deemed sarcopenic

based on muscle mass alone. This highlights the need for other tests such as muscle strength and function to be carried out alongside muscle mass assessment for the identification of sarcopenic individuals.

Figure 6.3.6.3. explores the differences between young and old, male and female in appendicular lean mass (ALM) and appendicular skeletal muscle index (ASMI) corrected for either by height (Figure 6.3.6.3 C & D) or BMI (Figure 6.3.6.3. E &F).

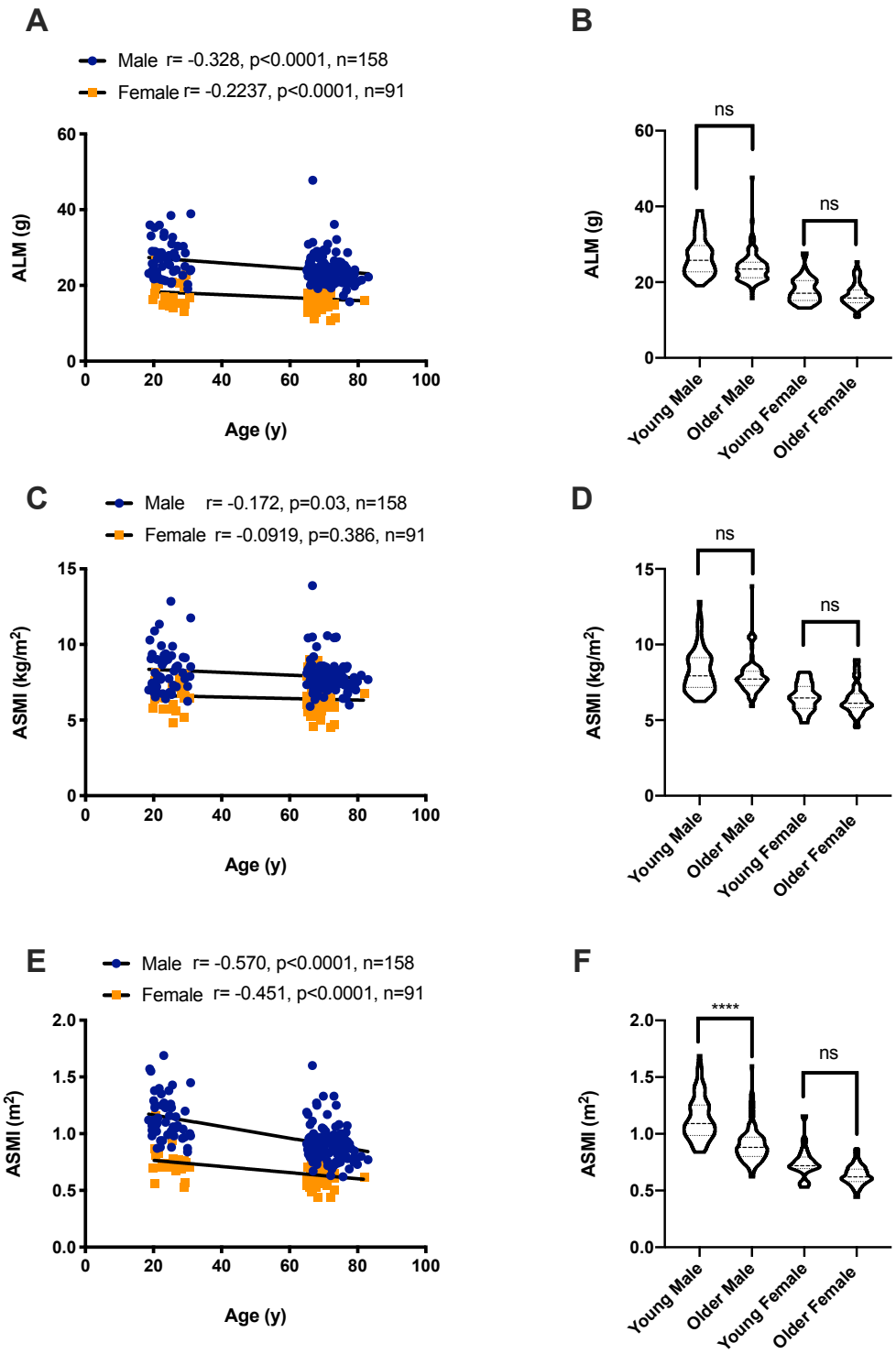


*Figure 3.3.6.3 The difference of appendicular lean mass (ALM) (A and B), appendicular skeletal muscle index (ASMI: i.e. ALM/ht^2) (C and D) and appendicular lean mass corrected for BMI (E and F) between young and old, male and female. **** denotes p<0.0001*

3.3.7.Application of different handgrip strength cut-offs to our cohorts

Based on a study by RM Dodds et al. 2014, that investigated handgrip strength in 49,964 participants (aged 4-90 years) from 12 British studies, the EWGSOP2 revised their original cut-off points for handgrip strength (Dodds *et al.*, 2014). The cut-off values have been selected based on gender specific T-scores of less than or equal to -2.5 (Cruz-Jentoft *et al.*, 2018). The use of T-scores was based from the way in which osteoporosis is identified from BMD data (Diamond and Sheu, 2016). Thereby, for handgrip strength the T-score was calculated as the individuals grip strength as a factor of the number of standard deviations below the peak mean of the gender matched young (Dodds *et al.*, 2014). As such the EWGSOP2 have updated the handgrip cut-off to 16kg from 20kg. This reduced the number of females in our older cohort who would be identified as sarcopenic based on strength by 50% (from 4 to 2 participants). For the males the EWGSOP2 updated handgrip strength from 30kg to 27kg cut-off reduced the number of male participants identified in our cohort as sarcopenic based on strength by 75% (from 8 to 2 participants). The FNIH use handgrip cut-off values of <26kg for men and <16kg for women. Therefore, the cut-off values now match the updated EWGSOP2 definition for women but are 1kg less than the EWGSOP2 in the cut-off for men. In our cohort, the number of participants identified as sarcopenic based on handgrip strength was the same for the FNIH and EWGSOP2 definitions. The IWGS do not provide a cut-off value for handgrip strength and only suggest gait speed as a

measure of physical function, therefore, this definition could not be compared.

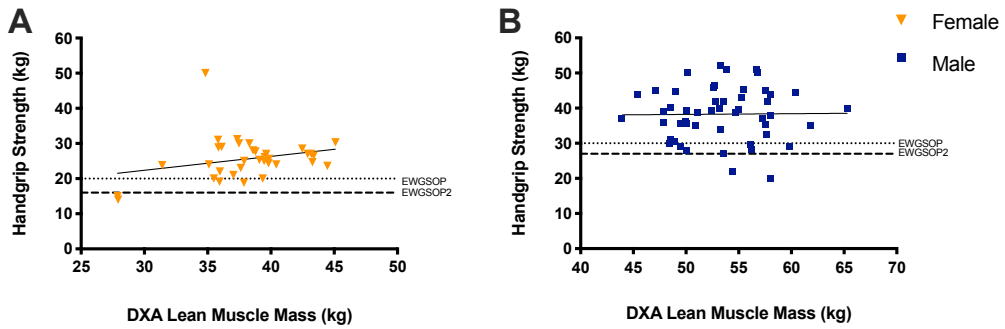


Figure 3.3.7.1 The updated EWGSOP2 cut-off point vs the original EWGSOP cut-off in females A) and males B).

3.3.8. Application of different functional physical performance cut-offs

For the assessment of muscle function, the EWGSOP, EWGSOP2, FNIH and IWGS suggest the measurement of gait-speed as this is a measure that can be easily performed in a clinical setting and with the elderly. Applying the EWGSOP gait speed cut off of <0.8m/s to our older cohort, 2 males and 1 female would classify as sarcopenic based of mobility. However, applying the IWGS gait speed cut off of <1 m/s to this cohort would result in 12 males and 6 females defined as sarcopenic. The SPPBT is another test suggested by the EWGSOP that can be used to assess muscle function. Combining these two main measures that are used in the assessment of sarcopenia, a greater number of participants would be deemed sarcopenic if using the cut-off for gait speed over handgrip strength. See Figure 3.3.8.1. Only 2 males equalled the 8-point SPPBT cut-off for the sarcopenia.

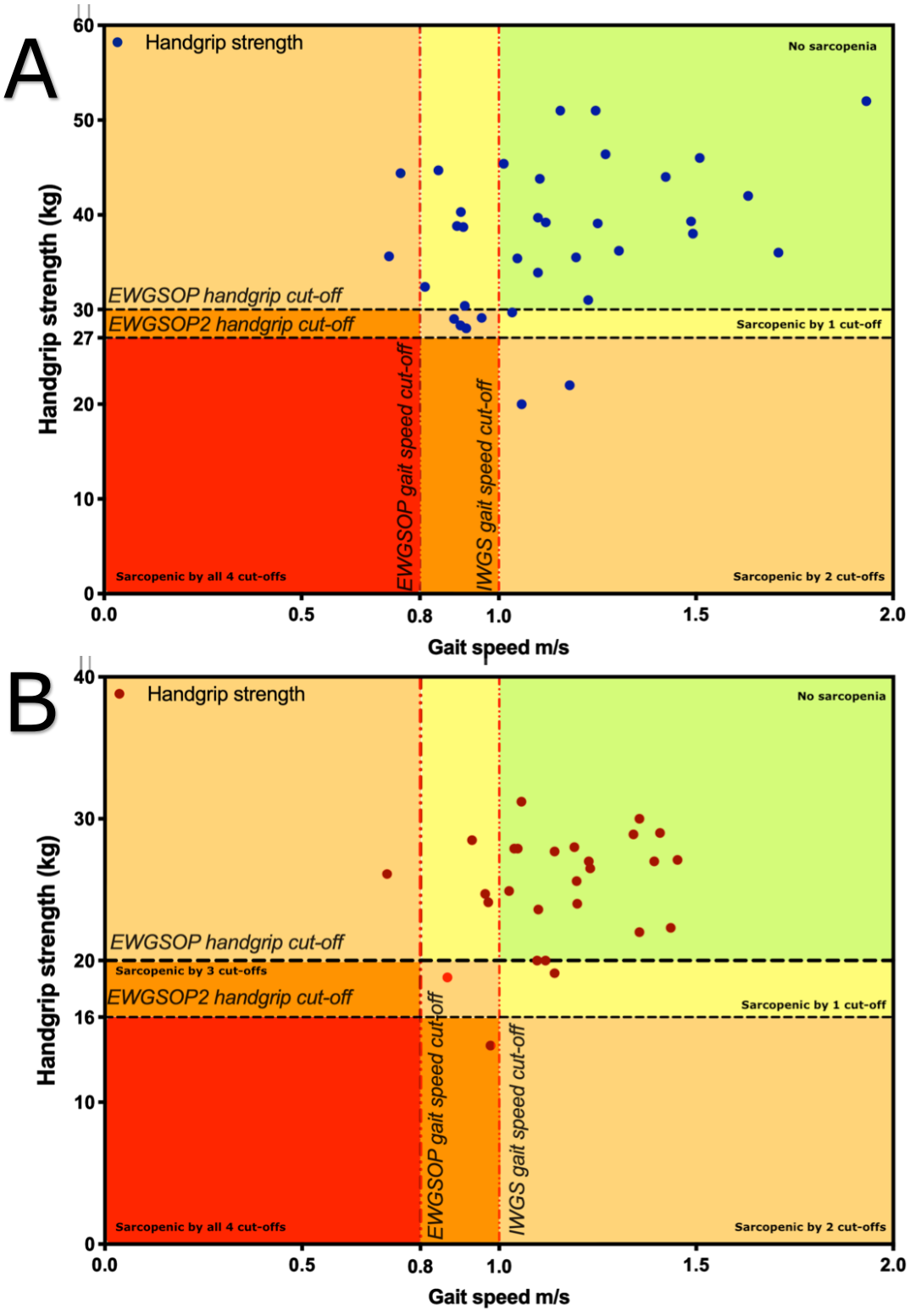


Figure 3.3.8.1 Scoring for handgrip strength vs gait speed for older males (A) and older females (B)

3.4. Discussion

It was only very recently that sarcopenia was finally recognised in the International Classification of Diseases, tenth revision, clinical modification [code: M62.84;(Anker, Morley and von Haehling, 2016)] with numerous definitions and clinical approaches for the identification of sarcopenia now existing. For a greater understanding in potential treatment or mitigation of sarcopenia, correct identification of sarcopenic individuals is crucial for both research and the clinical population; two populations that may differ in terms of muscle mass, muscle function and general health. Sarcopenia is associated with an increased risk of frailty (Bernabeu-Wittel *et al.*, 2019), falls (Scott *et al.*, 2014) and physical disability (Janssen, Heymsfield and Ross, 2002). Identifying sarcopenic individuals, ideally before diagnosis, will aid appropriate interventions that may help mitigate sarcopenia or prevent sarcopenia from occurring being prescribed to individuals. This could ultimately reduce their risk of frailty and falls and consequently provide individuals with a better quality of life. Current interventions suggested to treat sarcopenia are exercise, increased protein feeding with potentially leucine supplementation, vitamin D supplementation to also reduce the risk of osteosarcopenia and potentially testosterone provision (Anker, Morley and von Haehling, 2016). Yet currently no coherent definition for clinicians to identify or treat sarcopenia exists.

The aims of this study were two-fold; 1) to characterise the healthy older population participating in research studies in terms of general health, bone mineral indices, muscle mass and muscle function 2) to

compare the definitions currently used to define sarcopenia and how they impact on the identification of sarcopenic individuals. Herein the effect of applying different definitions to the same cohort of individuals via measurement of muscle mass, muscle function and strength has been investigated. Additionally, the variability of the prevalence of sarcopenia identified in our cohorts.

Characteristics of the older participants in research studies

The volunteers from these studies were separated by age and gender. Age was separated as 18-35y for the young and based on 'the EWGSOP' (Cruz-Jentroft et al., 2010), 65 y and over for the older cohort. Since we recruit "healthy" older individuals, our population may be defined as being pre-selected and therefore will be biased towards healthy elderly. It is likely not to be representative of the wider 'unhealthy', frail and clinical populations, especially as our selection criteria rules out a significant number of metabolic and non-communicable diseases. Specifically, when trying to identify sarcopenic individuals as sarcopenia exists alongside numerous chronic diseases e.g. diabetes (Morley *et al.*, 2014), COPD (Jones *et al.*, 2015), cancer (Villaseñor *et al.*, 2012). In the Health, Ageing and Body Composition study, consisting of 1,840 volunteers who were studied at baseline and with a 3-year follow-up, ~16.6% presented with type 2 diabetes and showed greater declines in muscle strength and muscle mass (Park *et al.*, 2007). In a study of 622 outpatients with stable COPD, ~15% were identified as sarcopenic (Jones *et al.*, 2015) by the EWGSOP criteria. Chronic diseases such as cancer,

COPD, thyroid disease and diabetes are all part of the exclusion criteria for healthy volunteers. Therefore, all our participants had blood chemistries within "normal" range. However, ~14.6% of the males and ~12% of females, in our older cohorts, would be classed as having stage 3a chronic kidney disease (i.e. glomerular filtration rate (GFR) between 45-59 mL/min/1.73m^2)(National Clinical Guideline Centre, 2014). In the UK, the overall prevalence of CKD (stage 1-5) is approximately 13%, which is inclusive of all adults (18+ years) (National Clinical Guideline Centre, 2014). However, the NEOERICA study standardised CKD prevalence by age and found that in their study population 17.7% of males (n=6356) and 27.9% of females (n=6943) aged between 65-74 years presented with CKD stages 3-5 (Stevens *et al.*, 2007). Therefore, our cohort may be reflective of the UK population for CKD.

Although we aimed to recruit healthy volunteers, there were significant age-related changes in body composition. The older volunteers had a significantly greater percentage fat mass than the younger cohort (males: 29 vs 22%, females: 39 vs 34%, respectively), which is consistent with the results from longitudinal and cross-sectional body composition studies (Kyle *et al.*, 2001; Hughes *et al.*, 2002). Declines in muscle mass have also been observed beginning from around the age of 30 (Forbes and Reina, 1970). Here, as expected, LBM was significantly lower in the older group than the young, in concordance with previous studies (Lexell, Taylor and Sj, 1988; Evans and Lexell, 1995; Frontera *et al.*, 2000).

Additionally, a significant negative regression was evident within the older males 65-85 y for LBM and age. However, this was not apparent within the older female group. This may be due to the age distribution within the older female cohorts, since 99% of the older female participants were between 65 and 74 years old, with only one participant over 80.

The loss of bone mass with age is well-established, with women experiencing a greater loss than men (Riggs *et al.*, 1981). In the present study a significantly lower BMD in older participants was recorded. BMD is the BMC corrected to the size of the bone (Deng *et al.*, 2002). BMC was significantly lower between the young and older females but not in males. As expected, BMD was higher in men than women. Within the older cohort, both males and females showed lower BMC, but not BMD, with age. Therefore, the changes in BMC are likely to be due to height or bone size differences. Furthermore, LBM had a greater correlation with BMD than percentage fat mass in both the older males and females, supporting the findings from a recent meta-analysis (Ho-Pham, Nguyen and Nguyen, 2014).

The relationships between BMD and muscle strength and ageing has been investigated, identifying muscle strength as a good predictor of BMD in both men (Snow-Harter *et al.*, 1992) and women (Snow-Harter *et al.*, 1990). As bone and muscle tissues interact together, the loss or disuse of one is associated with a decline in the other (Bevier *et al.*, 1989; Fricke *et al.*, 2010; Tarantino *et al.*, 2015). Here,

lower BMC correlated with lower handgrip strength and lower single leg 1RM extension only in the older females, but BMD only correlated with 1RM single leg extension. This may be as a consequence of women experiencing a greater decline in bone mass with age than men (Riggs *et al.*, 1981).

Whole-body LBM mass correlated with single leg 1RM extension but not handgrip strength in older males or females. A comparison between arm LBM and handgrip strength would need to be performed. As whole body changes may not reflect upper limb changes – that might correlate better with hand-grip changes, since lower limb mass and strength are lost at a greater rate than upper limbs with age (Candow and Chilibeck, 2005). Therefore, the relationship between upper limb strength and whole-body LBM may not be evident. Despite this, declines in upper body muscle strength with age were evident for the older females and older males for handgrip strength and gait speed. No significant correlations were identified between single leg MVC and single leg-1RM leg extension. In this study, there was a lower number of participants who had their single leg MVC assessed in comparison with the other 3 measures. In this study handgrip strength and walking speed may be greater predictors of loss of muscle function/strength with age than single leg 1RM knee extension or MVC.

Application of different definitions of sarcopenia to the research population

This data represents a cohort of healthy volunteers enrolling into research studies. However, when applying the different definitions of the EWGSOP (original and updated), IWGS and FNIH, there are clear discrepancies in the proportions of our volunteers who are deemed sarcopenic based on muscle mass alone. This is in line with recent findings by Mayhew et. al. (2019), who identified a range of 24.2 to 40.4% in the prevalence of sarcopenia in studies that utilised muscle mass as the only cut-off and did not include strength or function testing. In this study, the percentage even varied within the same definition depending on which calculation for muscle mass selected (e.g. ALM or ASMI adjusted for BMI). As mentioned previously, the ICD-10 does not have clear clinical guidance on which consensus on sarcopenia to use. This study identified a variability in prevalence of sarcopenia (older males: 5-23% and older females: 6-33%) when only focussed on muscle mass. This supports the investigations of recently published studies that also found the greatest prevalence of sarcopenia in muscle mass alone was identified using the EWGSOP (Mayhew *et al.*, 2019; Reiss *et al.*, 2019).

A recent meta-analysis identified that the EWGSOP criteria is the most used definition for the identification of sarcopenia (Mayhew *et al.*, 2019). The EWGSOP have updated their consensus following on from emerging research on sarcopenia over the ~10-year period since the initial consensus. The cut-off values for the assessment of low muscle

quantity by ASMI (ALM/ht^2) have been reduced from <7.26kg/m^2 to <7.0kg/m^2 for men and remained the same for women at <5.5kg/m^2 [if using the values by the Rosetta study (Baumgartner *et al.*, 1998)]. The EWGSOP2 have selected these cut-off values based on the appendicular lean mass reference ranges for Australian men and women from The Geelong Osteoporosis Study (Gould *et al.*, 2014). Although, how the EWGSOP determined the cut-off values is unclear as they do not correspond to the ASMI values presented in The Geelong Osteoporosis Study. Despite only a 0.26kg/m^2 change in the ASMI for men in the recent update, a decrease of 50% in prevalence of sarcopenia in older men has been observed between the EWGSOP and EWGSOP2 (Reiss *et al.*, 2019). Whereas this change only signified an 8% decrease in sarcopenic older males from our cohort. Therefore, this change in cut-off values may alter the findings of all previous literature that have used the original EWGSOP criteria by overestimating the number of male individuals identified as sarcopenic. For example, Jones et al. (2015) identified 15% of patients with COPD as sarcopenic using the original EWGSOP may now find a reduced percentage if the updated EWGSOP2 is used. The alternate measure for low muscle mass by the EWGSOP2 is the use of ALM which corresponds with the values provided by the FNIH as this is where the cut-off values are referenced from. However, the EWGSOP rounded the cut-offs to the nearest kilogram (i.e. from 19.75 kg to 20 kg in men and from 15.02 kg to 15 kg in women). Here in this study the change in cut-off for men was enough to

increase the prevalence of sarcopenia in older males by 3% from the FNIH definition to the EWGSOP2 consensus based on ALM.

The application of the different algorithms for the quantification of muscle mass in this study, identified 31% of the younger males from our cohort as sarcopenic. The only definition that identified 0% of the young males and females as sarcopenic based on muscle mass was the use of ALM adjusted for BMI provided by the FNIH. As it is unlikely that the younger cohort are sarcopenic, the use of ALM adjusted for BMI may be the more appropriate algorithm to use for the quantification of low muscle mass in sarcopenia. Mayhew et al. (2019) identified 109 studies only muscle mass for screening of sarcopenia. Therefore, in this study, the use of a young cohort alongside the older cohort allowed for comparisons and contradictions to be investigated. Although, currently there is debate about which algorithm for low muscle quantity is to be used whether its ALM adjusted for by height, weight or BMI (Cruz-Jentoft *et al.*, 2018). Here the younger cohort were used as a comparison as studies have been using single measurements of muscle mass to assess for sarcopenia as identified by Mayhew et. al. (2019). As a comparison, the cut-off values for ASMI (adjusted by height) used by the EWGSOP2 is based on t-scores formulated from a single study of 1,411 men and 960 women aged 10-93 years (Gould *et al.*, 2014), whereas, the cut-off values for handgrip strength are formulated from a combination of 12 different studies with a collective number of 49,964 participants (Dodds *et al.*, 2014). For a more robust determination of low muscle quantity, a similar approach to the determination of t-scores for

handgrip strength should be made, such as, combining muscle mass measures from different studies using ALM corrected for by height vs weight vs BMI.

Although, the inconsistency between the different definitions for muscle mass cut-offs and the effect of prevalence is known, this present study further investigates the contradictions and changes within the functional assessments, their cut-offs and the effect on sarcopenia prevalence. Handgrip strength is a good predictor of disability in older age (Giampaoli *et al.*, 1999; Bohannon, 2008).
The grip strength cut-offs by the EWGSOP2 are more closely aligned with FNIH for handgrip strength and in this study identified the same number of individuals as sarcopenic. However, the IWGS do not use handgrip strength as a muscle strength assessment for sarcopenia, and only determine muscle function by gait speed; therefore, they were not able to be compared. Difference between handgrip and gait speed numbers sarcopenic, if gait speed is fine and handgrip is not, may be missing out on sarcopenic individuals based on order used in clinical paradigm.

Study limitations

A limitation to this study was the inconsistent muscle function assessments used across the studies which limited the number of individuals who could be compared or identified as sarcopenic. A number of the studies (Phillips *et al.*, 2013, 2015; Matthew S. Brook *et al.*, 2015; Wilkinson *et al.*, 2017) from which the data for this study

has been obtained, were recruited before the introduction of SARC-F. The number of different cut-offs for muscle assessments is large. This study only observed DXA cut-offs, but calf-circumference has been suggested by the EWGSOP2 and BIA is an alternative measure.

Future Directions

Future researchers should be clear about the definitions used and be aware of the differences between cut off values chosen. A general consensus agreed internationally should be formulated to align with the ICD-CM-10 code to provide a clearer definition for all to follow consistently. For researchers studying muscle mass, strength or function in older adults, utilising a uniform muscle assessment would aid with better cross-study comparisons and the potential for future collation of data in the creation of population-specific reference values. Furthermore, there are no "at-risk of sarcopenia" muscle mass or muscle function values. These should be developed similar to the T-Scores used for BMD to aid prevention of sarcopenia.

Conclusions

There is no consistency between the 4 main definitions of sarcopenia in the quantification of low muscle mass or assessment of low muscle strength or physical function. Applying the sarcopenia cut-offs to our past healthy older and younger research participants who are not representative of the whole UK population (apart from CKD stage 3a incidence), still managed to identify sarcopenic individuals based on muscle mass or physical function; even within the younger cohort.

Despite the EWGSOP recently releasing an updated definition, this has brought in greater disparity in the identification of sarcopenia which needs to be resolved to aid clinicians or researchers in correct diagnoses.

4. Development of combined minimally invasive stable isotope approaches to identify any relationships and simultaneously measure muscle protein turnover and muscle mass

4.1. Introduction

An alternative measure of whole-body muscle mass is the use of D_3-creatine (D_3-Cr). The labelled creatine will equilibrate within the creatine pool, of which approximately 98% can be found in skeletal muscle (Hunter, 1922). Creatine is converted to creatinine through an irreversible enzymatic reaction. Creatinine is transferred from the muscle to the kidney where it is filtered and can be found in urine (Markus Wyss and Kaddurah-Daouk, 2000). After ingestion of a known amount of D_3-Creatine, the total amount absorbed can be calculated from the quantification of the labelled creatine concentration found in a subsequent 24-hour pooled urine sample. It can take up to 40-50 hours for the creatine to fully equilibrate within the body. Single urine samples taken at 48 and 72 h post-ingestion of D_3-creatine are analysed for the ratio of labelled to unlabelled creatinine. From this the creatine pool size can be quantified and based on 4.3g creatine/ kg of muscle (Baldwin *et al.*, 1952; Barnes, Gordon and Cope, 1957; Fitch and Sinton, 1964), the total whole-body muscle mass can be estimated. The use of isotopically-labelled creatine to measure muscle mass was first described in the late 1960's (Meador et al. 1968, Kreisberg et al. 1970). In 2014, Clarke et al. (2014) published their developments and validation of the D_3-Creatine method; identifying a strong correlation between muscle mass measured by the D_3-creatine method and muscle mass measured by MRI in humans.

D_3-3-methylhistidine (D_3-3MH) has recently re-emerged as a technique to measure whole-body muscle protein breakdown (MPB). 3-methylhistidine is formed post-translationally, from the methylation of histidine residues in protein, and released as a result of contractile muscle protein (actinomyosin) breakdown, particularly from degradation of the actin fragment (Asatoor and Armstrong, 1967). The oral provision of stable isotopically labelled D_3-3MH can be used to assess the rate of this whole-body MPB via plasma or urine sampling over time and measuring the rate of dilution of D_3-3MH by endogenous 3MH release (Sheffield-Moore *et al.*, 2014). In comparison with existing measures of muscle protein breakdown that require intravenous infusions, the use of multiple tracers or muscle biopsies, the use of D_3-3MH provides a promising minimally invasive alternative that may be used to assess MPB across a wider range of populations (e.g. clinical, frail or in care-home).

Deuterium oxide (D_2O) is already a well-established stable-isotope tracer technique that can be used to measure muscle protein synthesis (MPS). Although a muscle biopsy is still required for the determination of muscle synthetic rate, the use of D_2O is much less invasive in comparison with traditional stable isotope labelled amino acid (AA) tracer techniques. Unlike the traditional AA techniques, D_2O does not require blood or muscle sampling for the determination of precursor enrichment, intravenous tracer infusions requiring multiple biopsies or cannulation (Wilkinson *et al.*, 2015).

The combination of all three methods to develop a minimally invasive stable isotope approach to measure MPS, MPB and muscle mass simultaneously has not previously been performed. This chapter will explore the development of each method and the design of a protocol that combines all three measures to provide an approach that can assess MPS, MPB and muscle mass in a minimally invasive fashion, firstly in 10 older males as a pilot.

4.2. Methods

4.2.1. Development of the D_3-Creatine method

4.2.1.1. *Dosing and Sampling*

Based on the method by Clark et. al (2014) Subjects were asked to provide a single urine "spot" sample before ingesting 30 mg of D_3-Creatine (CK Isotopes) dissolved in 50 ml of double-distilled water (ddH_2O). Following ingestion of the tracer, subjects were provided with two 3L containers for all urine collection over the successive 24h. After an initial morning void, single spot urine samples (50ml) were collected at 48 and 72h after tracer ingestion. Aliquots (1ml) of the urine were taken and stored in eppendorfs at -80˚C. Total volume of 24h collection was recorded, before an aliquot was taken. Urine samples were prepared for analysis on the Liquid Chromatograph-Mass Spectrometer (LC-MS) as previously described in Chapter 2.

4.2.1.2. *Preparation of samples*

The preparation of the urine samples is described in Chapter 2. A standard curve was prepared in order to quantify the amount of D_3-

creatine that was not taken up by muscle, but simply excreted in the urine during the first 24h after ingestion. ^{13}C-Creatine (M+1) was used as an internal tracer for quantification, as it would not interfere with the measurement of D_3 creatine (M+3). 10µl of ^{13}C-Creatine was added to 50 µl of urine. For the determination of D_3-creatinine enrichment, to determine creatine pool size, an enrichment curve from 0-0.1% of D_3-creatinine was run alongside each batch.

4.2.1.3. *LC conditions*

LC-MS conditions were based on a method previously described by (Leonard, Dunn and Smith, 2014). 2µl of sample was injected into the Agilent 1100 series liquid chromatograph that was connected to the Thermo Scientific Q Exactive Mass spectrometer. The flow was set to 0.2µl/min, 60:40 (Buffer A:B) isocratic flow; where Buffer A was 100% Acetonitrile LC-MS grade and Buffer B Ammonium Acetate pH 5.8.

4.2.1.4. *MS conditions*

Polarity was set to positive with a resolution of 70,000. A full scan method identified the masses of D_3-creatine, ^{13}C-creatine and ^{12}C-Creatine as 135.09549, 133.08006 and 132.07666, respectively. The same full scan method was performed to identify ^{12}C-creatinine and D_3-creatinine where the mass was identified as 114.06638 and 117.08515, respectively. The identified masses were used as inclusion masses for the SIM (selected ion monitoring) method for creatine and creatinine. Peak areas were integrated using Xcalibur software (See Figure 4.2.1.1) for the calculation of isotope ratios and then concentration and enrichment.

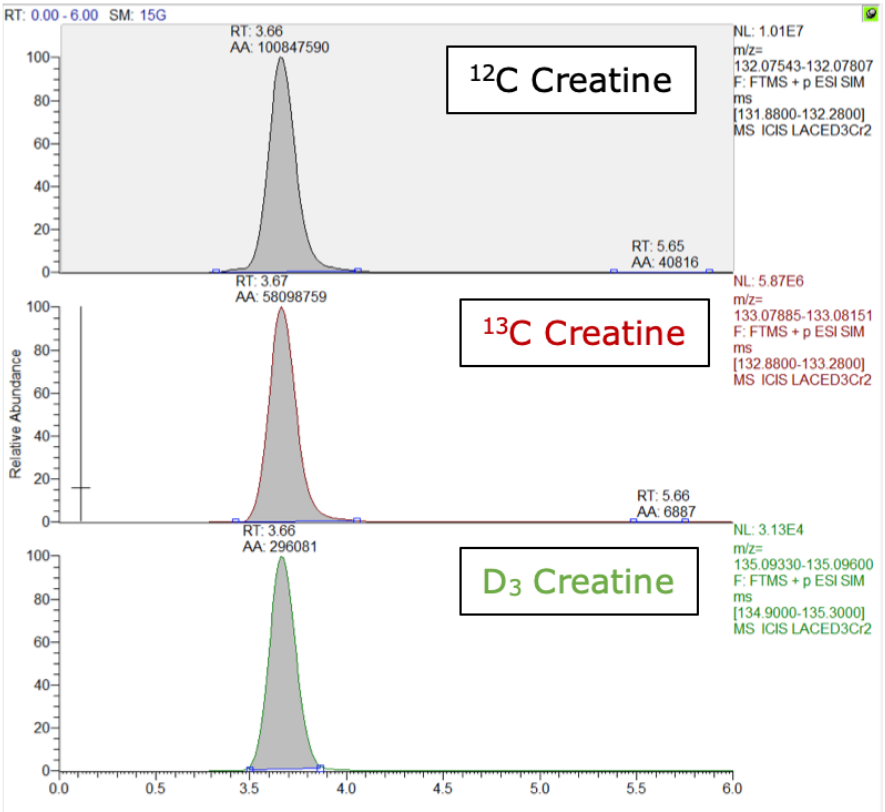


Figure 4.2.1.1 Example chromatograms for the determination of ^{12}Creatine, ^{13}Creatine and D_3Creatine

4.2.1.5. *Calculation of muscle mass*

Muscle mass was calculated using the following equation:

$$Total\ muscle\ mass = \frac{\left(\frac{MW_{Unlabelled\ Cr}}{MW_{labelled\ Cr}}\right) \times (Amount\ of\ D_3{-}Cr\ dosed\ (g) - Amount\ of\ D_3{-}Cr\ excreted\ (g))}{(mean\ steady{-}state\ D_3{-}Crn\ enrichment\ ratio\ )} \div 4.3(g/kg)$$

(Clark *et al.*, 2014)

Where MW unlabelled Cr is the molecular weight of creatine of 131.1 and MW labelled Cr represents the molecular weight of D3 creatine i.e. 134.1. The amount of D_3-Cr excreted in the 24h is subtracted from the amount dosed to correct for the amount absorbed. 4.3 g/kg represents the concentration of creatine per kilogram of wet muscle. The creatine pool size that is calculated is divided by this factor to calculate a total muscle mass.

4.2.1.6. *Statistical Analysis*

All data sets were tested for normality. All statistical analysis was performed using Graphpad Prism software (Prism 8, USA).

4.2.2.Development of the D_3-3-methylhistidine method

4.2.2.1. *Dosing and Sampling*

Dosing was based on the study by Sheffield-Moore et al. (2014). A single blood sample was taken before provision of 10 mg of D_3-3MH (CK Isotopes), dissolved in 50 ml of water. The following day (24 h post-tracer ingestion) volunteers had blood samples taken hourly from a cannula. Blood samples were centrifuged at 3,200 rpm for 20

minutes at 4°C. Subsequently, the plasma was aliquoted and frozen at -80°C until further analysis.

4.2.2.2. *Preparation of samples*

Plasma samples were prepared ready for analysis as described in Chapter 2. An enrichment curve of 0-10% D_3-3-methylhistide was prepared alongside each batch of samples.

4.2.2.3. *LC -conditions*

2μl was injected into the Agilent 1100 series liquid chromatograph connected to the Thermo Scientific Q Exactive Mass spectrometer. The flow was set to 0.4μl/min with an initial buffer gradient of 95:5 (Buffer A:B); where Buffer A was 10mM ammonium formate (90:10 Acetonitrile: ddH_2O) with 0.1% Formic Acid and Buffer B was 10mM ammonium formate (50:50 Acetonitrile: ddH_2O) with 0.1% Formic acid. After a 2.5 minute hold at 95:5 (A:B), the buffer gradient was ramped to 100% Buffer B and held for 2.5 minutes before returning back to 95:5 (A:B). This was held for 13.5 minutes to re-equilibrate the column.

4.2.2.4. *MS conditions*

Polarity was set positive and resolution was set at 35,000. After using a full scan method, the masses for D_3-3MH and 3MH were identified as 173.11131 and 170.09230, respectively. These masses were set up in a SIM method. Peak areas were integrated using Xcalibur software.

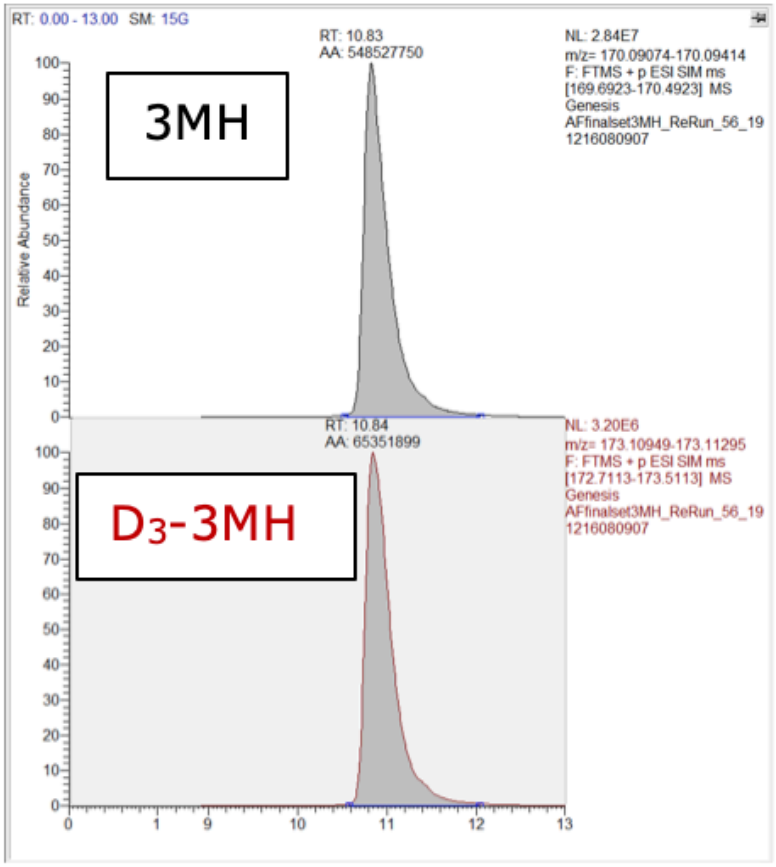


Figure 4.2.2.1 Example chromatographs of 3-methylhistidine and D_3-3-methylhistidine

4.2.2.5. *Calculation of muscle protein breakdown*

The ratio of D_3-3MH to 3MH was determined for each sample. The ratios were corrected relative to the enrichment curve, run with each batch, and the atoms percent excess (APE) determined. The pre-dose/baseline blood sample was used to correct for background and calculation of the APE. The ratios were log transformed and plotted against time to determine the rate of decay. See section 3.3.6

4.2.2.6. *Statistical analysis*

All data sets were tested for normality. All data are presented as mean ± SD.

4.2.3. Calculation of Muscle Protein Fractional Synthetic Rate using D_2O

4.2.3.1. *Dosing and Sampling*

With the measurement of muscle mass, using D_3-Creatine requiring up to 72h, MPS could also be measured over this time period to minimise number of visits and burden on the volunteer. Based on previous studies, 150 ml of D_2O (70% AP) was used to measure MPS over an acute period (3-7days), due to the long half-life of D_2O and sensitivity of the analysis, using IRMS. Saliva samples were taken pre-dosing then 2h post to identify the peak enrichment, when the label was fully equilibrated. Saliva samples were taken at 12h, 24h, 48h and 72h to calculate the decay of D_2O enrichment in body water over time and determine the precursor labelling throughout the measurement period. To reduce the sampling burden and minimize the invasiveness, a single blood sample, taken prior to the D_2O dose, could be used in D_2O naïve individuals to determine the background labelling alanine in blood/plasma protein. Therefore, in our protocol, a blood sample was taken pre- D_2O ingestion and a single muscle biopsy was taken at 72h.

4.2.3.2. *Preparation of Samples*

The preparation of saliva and muscle samples is described in Chapter 2. The blood sample was centrifuged at 3,200 rpm, for 20 minutes at 4°C. Plasma was aliquot and stored at -80°C. The plasma was deproteinised using ice-cold ethanol (100%) and centrifuged at 17,000g for 10 minutes. The pellet was transferred to a boiling tube containing on dowex with 0.1M HCl and hydrolysed, overnight at 110°C 16-20h; following the same preparation steps as the muscle sample (described in Section 2.)

4.2.3.3. *TC-EA conditions*

0.1 µl of sample was injected into the TC-EA (Thermo-Finnigan). The GC was set at 90°C and the reactor was set at 1400°C to ensure complete and efficient pyrolysis. Prior to the elution of the H_2 peak, from pyrolysis of the saliva water, two H_2 reference peaks (-202.45 delta H_2, compared to VSMOW (Vienna Standards Mean Ocean Water, an international standard, Fig 3.2.3.1). Peak ratios were determined via the ISOdat software.

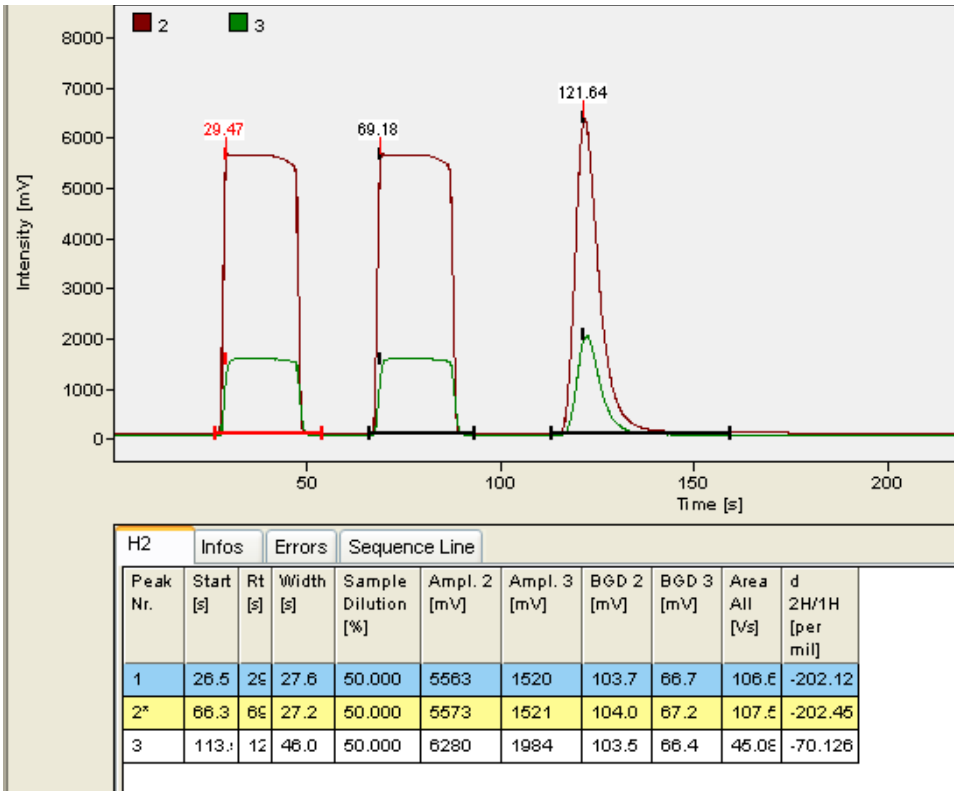


Figure 4.2.3.1 Example trace for two reference peaks and a sample with the red line identifying mass 2 and the green line mass 3.

4.2.3.4. *Calculation of Fractional Synthetic Rate*

From the saliva samples, the mean AUC of the APE was used to represent the average precursor labelling of free intramuscular Alanine, times 3.7 to account for deuterium exchange into alanine from D_2O, for the determination of muscle FSR. The calculation of myofibrillar fractional synthetic rate was based on the following product-precursor equation:

$$\textbf{FSR (\% /h)} = \frac{\Delta E_m}{(\Sigma E_p \times t)} \times 100$$

Where ΔE_m represents the change in enrichment of muscle i.e. from the plasma sample to the 72h muscle biopsy. ΣE_p represents the mean precursor enrichment and t represents the time over which the measure was taken. Using previously described equations by Brook et al. 2015.

4.2.4.Combination of the three approaches

For an initial blood sample, urine sample and saliva sample pre-tracer administrations, volunteers would be required to attend the clinical laboratory. To limit the number of lab visits required, as urine collection is required until 72 h, subjects continued urine collections up to 72h at home and returned at 72 h (day 3) with saliva and urine samples that were kept refrigerated (4°C). As volunteers would be returning, a single biopsy, for the MPS measurement was taken at this time in addition to cannulation for MPB measures. See Figure 4.2.4.1. Thereby, the assessment of MPS, MPB and muscle mass would only require 2 lab visits. If the blood sample could be taken in-home and DXA moved to the day of biopsy, this could reduce the study to one lab visit.

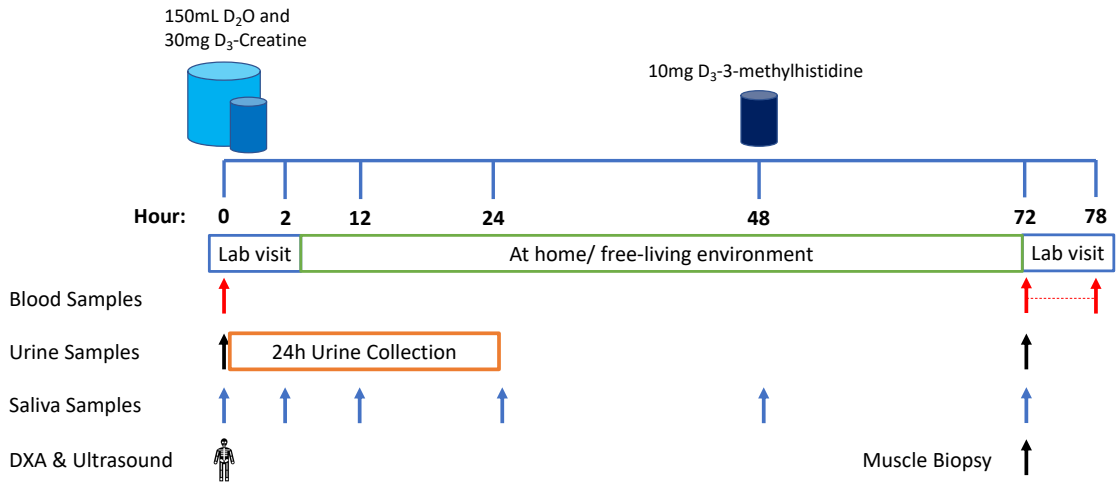


Figure 4.2.4.1 Protocol for combined tracer approach

4.2.5. Quantification of MPB in intervention studies using D_3-3-methylhistidine

The following section presents protocols from collaborative studies in which 3MH was analysed.

Bed-rest study: Study led by Tessa Morris-Paterson and Stephen Harridge (King's College London).

Twelve healthy young males (28 ± 4 years) underwent testing after a 7-day control phase and, then after a 6- week washout, again after 7-day supine unloading on a hyper-buoyancy floatation. The hyper-buoyancy flotation used was a hyper saline filled bed that would mimic a microgravity environment, where loss of muscle mass and function is experienced (Fitts, Riley and Widrick, 2001). After a baseline blood sample, 10 mg of D_3-3MH was ingested. Blood samples were taken 18-24h post tracer ingestion during the control phase (i.e. no bed rest) and at the end of the bed rest phase.

6-week resistance exercise training study: This study was run by Nima Gharadaghi and was conducted within our research lab and previously published (Gharahdaghi *et al.*, 2019).

Briefly, 18 healthy, non-hypogonadal older men who underwent a double-blind placebo vs testosterone study involving 6-weeks of whole-body resistance exercise training, in an attempt to promote muscle growth/hypertrophy. D_3-3MH was administered and blood samples collected on 3 occasions i.e. at baseline, 3 and 6 weeks of the study. The plasma samples were analysed 21-25h post 10 mg of D_3-3MH ingestion.

4.3. Results

4.3.1. Determination of D_3-creatine spill over

The ratio of D_3-Creatine to unlabelled creatine in the 24h urine was measured to determine the percentage (%) of the dose not incorporated into the body creatine pool. The greatest excretion of D_3-creatine was observed during the 0-24h time period (See Figure 4.3.1.1). The percentage of the dose not incorporated into the body creatine pool is used to correct the total dose absorbed and subsequent dilution. Therefore, the total volume of the 24 h urine collection was recorded, and the total creatine concentration of the 24-pool sample was quantified. The mean total 24h volume was 1.94 ±0.71L (±SD). Total 24h creatinine concentration was not measured to be able to assess completeness. See section 4.2.1.5 for muscle mass calculation. For comparison to previous literature, the median and range (min-max) are presented in Figure 4.3.1.2. The mean percentage dose excreted was 0.38%, median 0.065% in the older males. When comparing this to Clarke et al. (2014) who tested this method on 6 older males with 30 mg of D_3-Creatine, they found a much larger percentage dose excreted in the 0-24h sample, with a median of 2.06% but maximum of 12.6% (See Figure 4.3.1.2B).

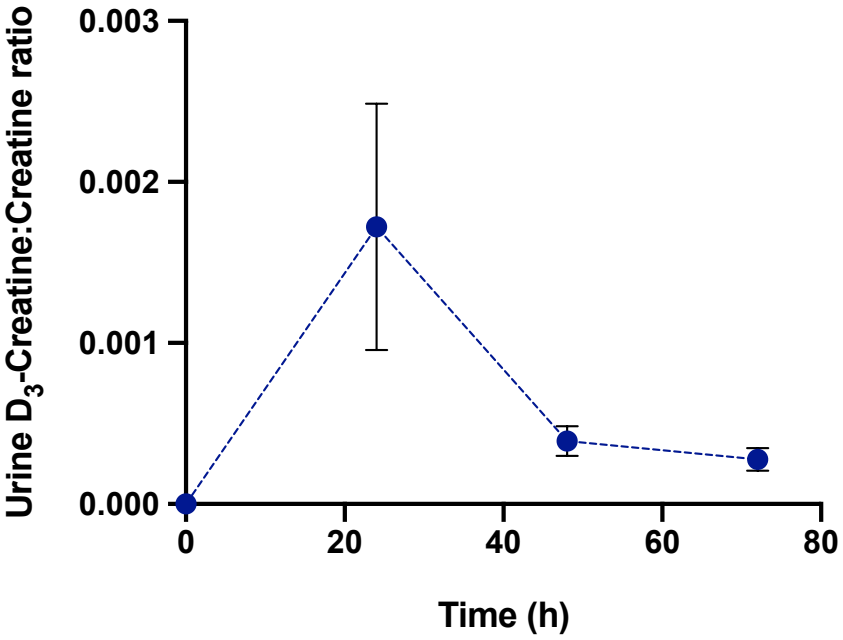


Figure 4.3.1.1 Urine D_3-creatine to unlabelled creatine ratio at time points 0, 24, 48 and 72h.

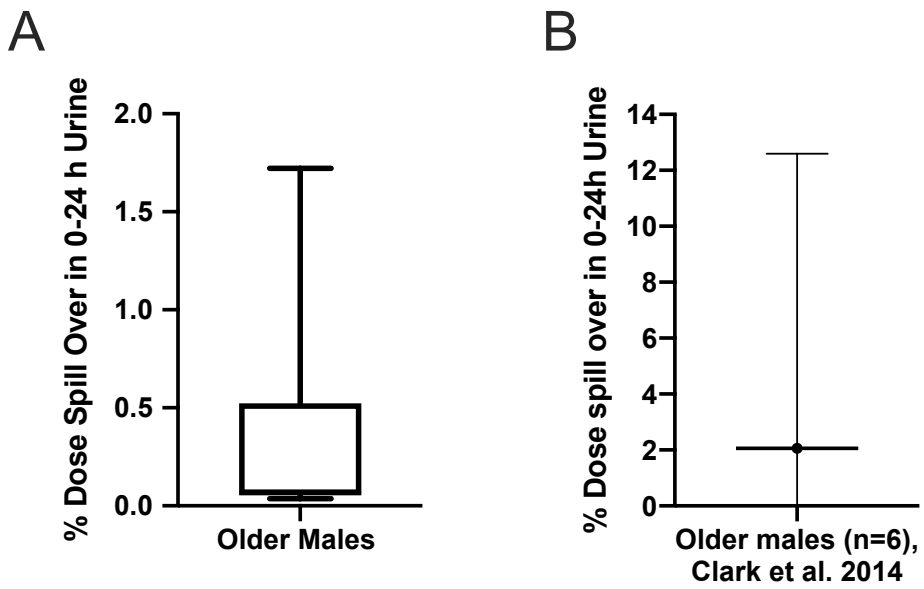


Figure 4.3.1.2 Percentage of Dose excreted in the 0-24 h urine in older males of this study (A) and older males from the study by Clark et al. (2014) (B). Values displayed as median and min-max.

4.3.2. D_3-creatinine enrichment

D_3-creatinine enrichment in the urine samples increased over time until 48 h where it plateau. There was no significant difference between the urine D_3-creatinine enrichment at 48 and 72h. The mean enrichment values of the 48h and 72h urine samples were averaged and used to calculate muscle mass.

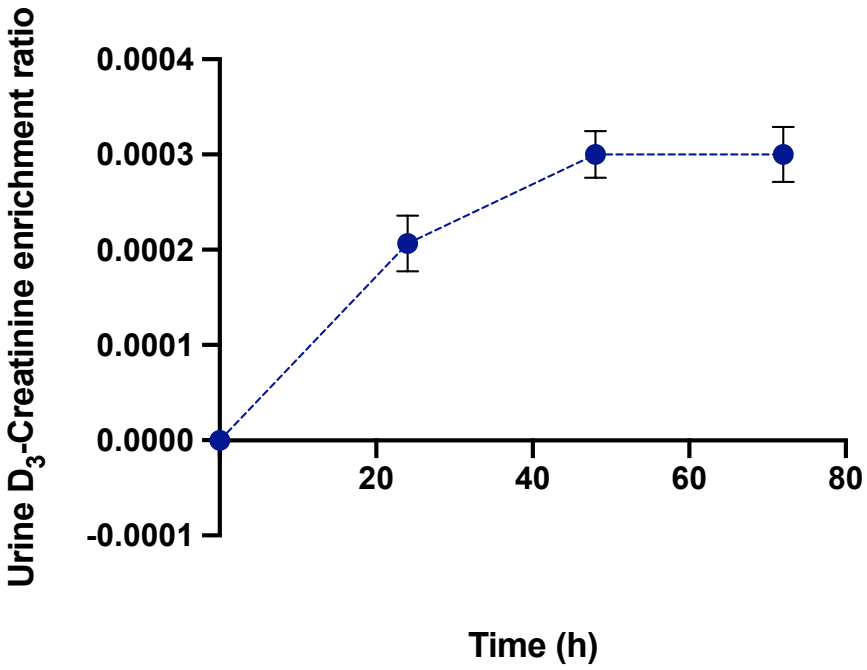


Figure 4.3.2.1 D_3-creatinine enrichment ratio corrected to baseline at 0, 24-pool, 48 and 72h.

4.3.3. D_3-creatine muscle mass quantification and correlation to ALBM from DXA

Lean body mass, appendicular body mass measured by DXA and creatine pool size and muscle mass estimates by D_3-creatine for this cohort are outlined below (See Table 4-1). There was no significant difference between ALBM and muscle mass measured by the D_3-

Creatine method (p=0.11, Paired t-test). Lean body mass (LBM) 53.2kg measured by DXA may be more representative of creatine pool size 98.8 g. There was a significant difference between LBM and muscle mass measured by D_3-Cr (p<0.0001).

	Older Males (65y+)
n	10
Creatine pool size (g)	98.8 ± 12.4
Muscle mass measured by D_3-Creatine (kg) (Cr pool size (g)/4.3 (g/kg)	22.9 ± 2.9
Lean body mass (LBM) by DXA (kg)	53.2 ± 4.9
Appendicular lean body mass (ABLM) by DXA (kg)	23.6 ± 2.6

Table 4-1 Creatine pool size and muscle mass measures for the older males (65y+). Mean ±SD.

4.3.4. D_3-creatine vs DXA

There was no significant correlation between LBM and muscle mass measured by D_3-Cr (r=0.55, p=0.098, r^2=0.31). However, ALBM was significantly positively correlated with muscle mass as measured by the D_3-Cr method (r=0.69, p=0.027, r^2=0.48). See Figure 4.3.4.1. DXA LBM had a significant positive correlation with ALBM (r=0.89, p=0.0011, r^2=0.88).

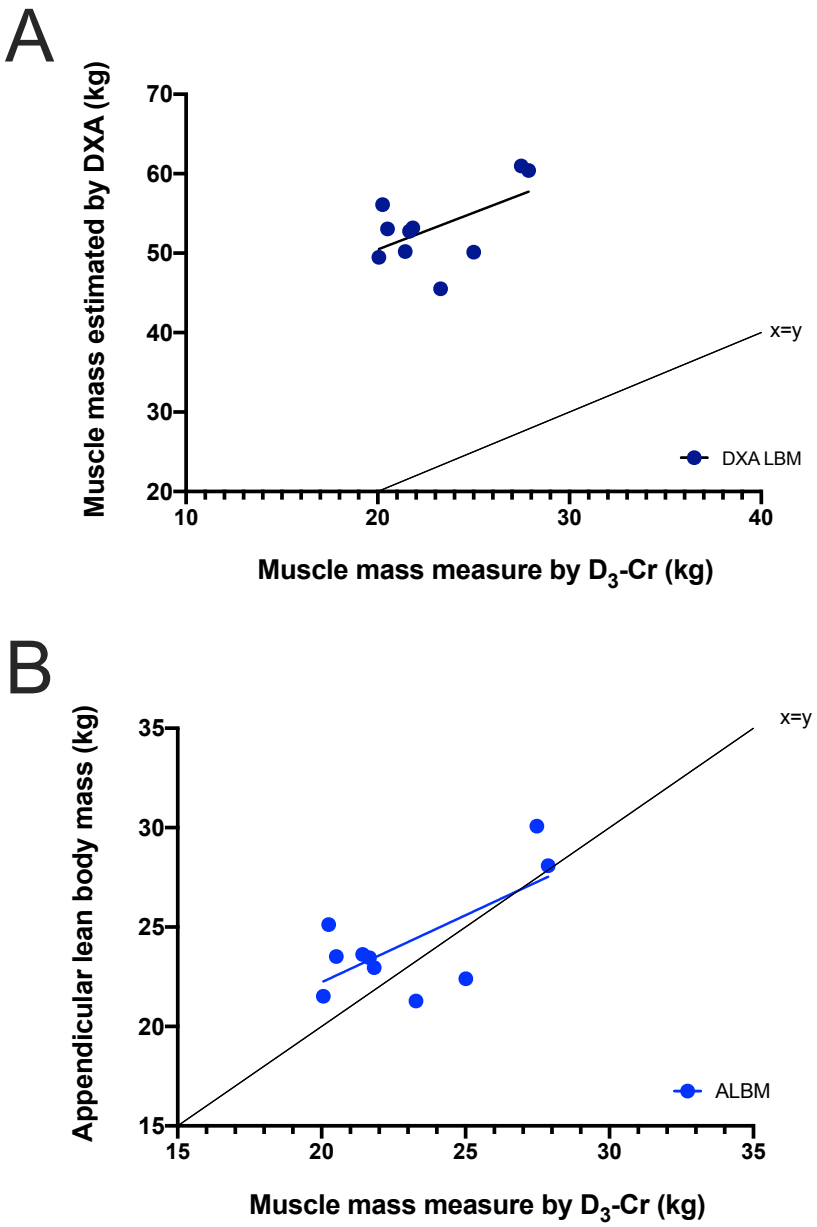


Figure 4.3.4.1 Muscle mass measured by D_3-Cr vs A) Total lean body mass (LBM) and B) appendicular lean body mass (ALBM) by DXA.

4.3.5. D_3-3-methylhistidine enrichment decay

Mean plasma D_3-3-methylhistidine enrichment gradually declined from 4.86 ± 2.25% APE at 24 h post- tracer ingestion to 3.65 ± 1.82% APE at 30h. See Figure 4.3.5.1.

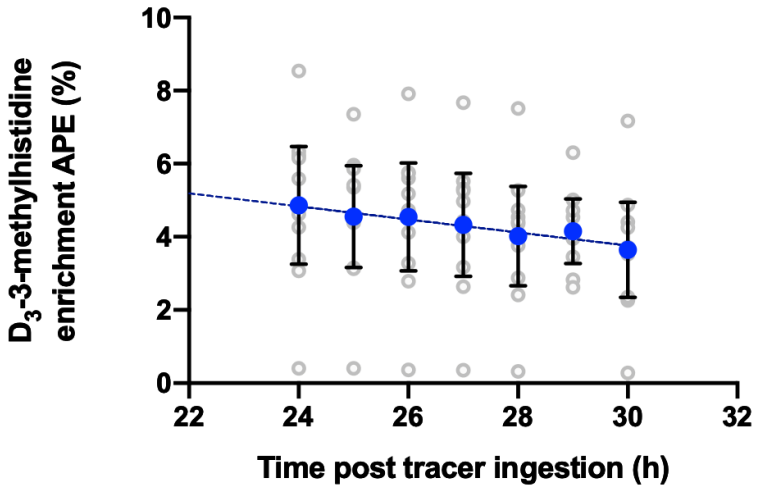


Figure 4.3.5.1 Mean D_3-3-methylhistidine enrichment in plasma from 24-30h post tracer ingestion (Mean ± 95%CI)

4.3.6. Determination of rates of MPB by D_3-3-methylhistidine

The ratios of labelled to unlabelled 3-methylhistidine were log transformed to determine the rate of whole-body muscle protein breakdown. Figure 4.3.6.1. The gradient of the straight line provides the k value. This was calculated for each individual. The mean rate of whole-body MPB for the 10 older males is 0.052 h^{-1}(95% CI, 0.038 to 0.067 h^{-1}); see Figure 4.3.6.2.

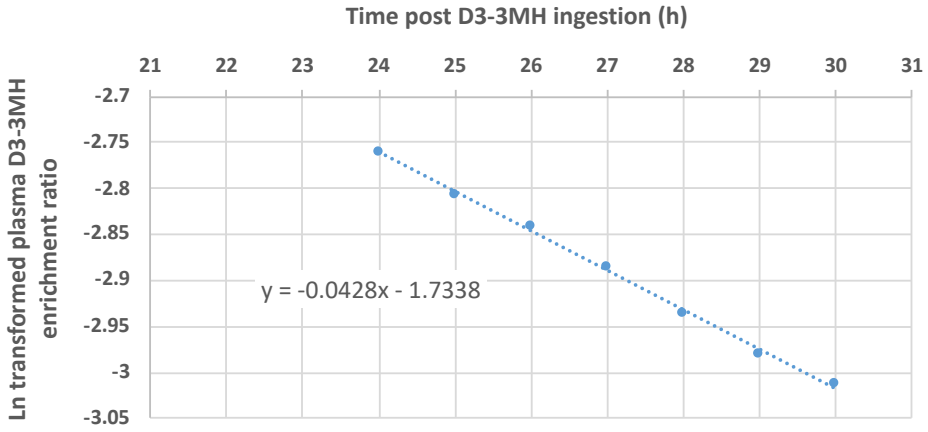


Figure 4.3.6.1 Example determination of the rate of muscle protein breakdown (k) from ln transformed plasma D3-3-methylhistidine enrichment ratio

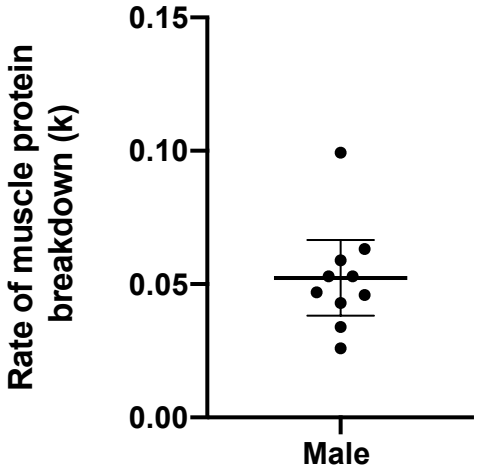


Figure 4.3.6.2 Mean rate of whole-body muscle protein breakdown determined by D_3-3-methylhistidine in 10 older males (Mean ± 95%CI).

4.3.7. Body water enrichment

The mean body water enrichment of D_2O peaked at 2h (0.24 APE; 95% CI, 0.23 to 0.26 APE) and significantly declined over time to 0.18 APE (95% CI, 0.16 to 0.20 APE) at 72 h.

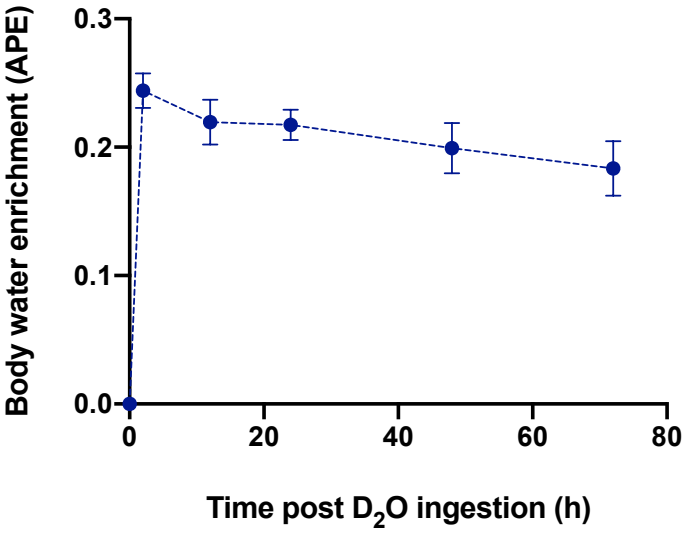


Figure 4.3.7.1 Mean body water enrichment of D_2O at 2, 12, 24, 48 and 72h (Mean ± 95%CI)

4.3.8. Myofibrillar Fractional Synthetic Rates

The mean myofibrillar FSR over 0-72h was 0.072 %/h (95% CI, 0.064 to 0.081 %/h). See Figure 4.3.8.1.

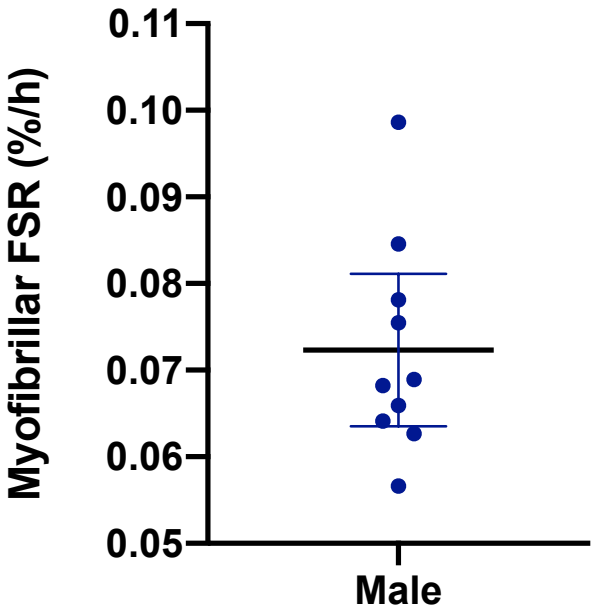


Figure 4.3.8.1 Mean rate of myofibrillar fractional synthetic rate (FSR). Mean and 95% CI.

4.3.9. Application of the D_3-3-methylhistidine approach to measure MPB before and after an intervention

The following section presents plasma samples analysed for 3MH from collaborative studies.

In a study led by Tessa Morris-Paterson and Stephen Harridge (Kings College London), we have further investigated the use of D_3-3MH tracer in the same 12 subjects who underwent a 7-day bed rest to identify any changes in whole-body MPB (data not yet published). From this preliminary data it was obvious that as a result of 7 days of bed rest whole-body MPB was suppressed (control phase: 0.062 ± 0.023k, bed rest: 0.043 ± 0.031k; mean ± SD). This is contrary to expectations that the loss of muscle that occurs during disuse/bed-rest is the result of increased MPB (See Figure 4.3.9.1).

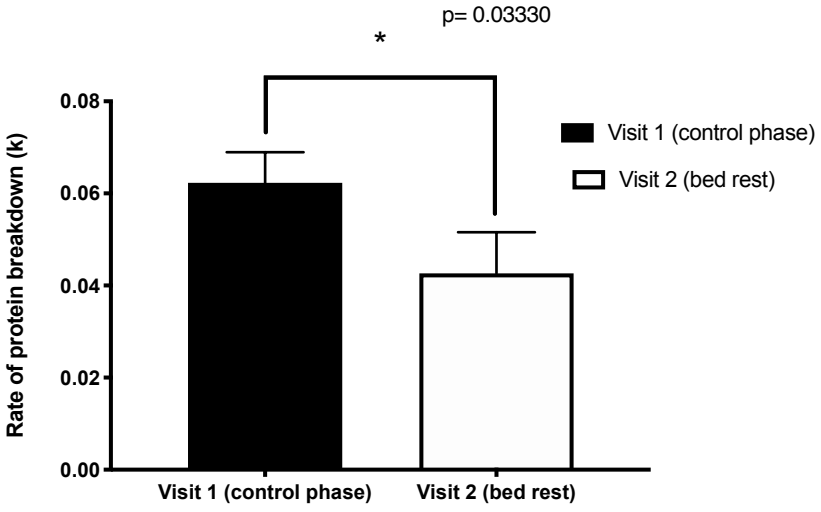


Figure 4.3.9.1 The effect of 7-day bed- rest on whole-body MPB rates measured by D_3-3MH (n=12, mean ± SD)

In the 6-week resistance exercise training study, there was no significant difference in whole-body MPB between the placebo or

testosterone group at baseline, 3 or 6 weeks. Although there was a slight non-significant increase in rate of MPB at 6-week vs BL in the testosterone group (P=0.11). See Figure 4.3.9.2.

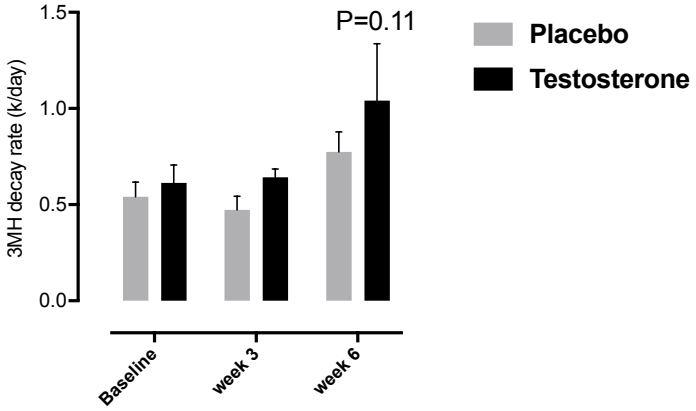


Figure 4.3.9.2 Whole-body MPB decay rate at baseline, week 3 and week 6 of the placebo and testosterone groups. (Mean ± SEM, Placebo: n=9, Testosterone: n=9)(Gharahdaghi et al.*, 2019).*

4.4. Discussion

Imaging techniques such as CT or MRI are considered the "gold-standard" for the measurement of skeletal muscle mass (Reeves, Maganaris and Narici, 2004). However, these techniques are typically expensive and not always easily accessible. Alternatively, DXA scanning is a commonly used technique to assess lean tissue mass, which can be mistaken as a proxy measure of muscle mass (Maden-Wilkinson *et al.*, 2013). DXA measures lean mass/ mass, which is a combination of muscle mass, organ tissue and water content (Evans *et al.*, 2019). As DXA is a commonly used approach to estimate muscle mass in the identification of sarcopenia, it was used in this study as a comparative measure in this study. The use of D_3-creatine can provide an alternative, minimally invasive technique (i.e. only oral tracer ingestion and urine sampling) to provide a direct estimate of muscle mass. This can potentially provide a more accessible

alternative to an estimate of muscle mass, albeit it may require access to expensive mass spectrometric equipment and skilled operators, it does however lend itself to batch analyses and ease of application and sample collection, which offsets some of the limitations.

Traditional AA stable isotope tracer methodologies used for the measurement of MPS and MPB are costly to undertake and require a clinician and appropriate clinical facilities/rooms given that they require intravenous infusions, arterial and venous cannulation and multiple biopsies, measurement of blood flow (Wilkinson *et al.*, 2015). D_2O is already a well-established stable-isotope tracer technique that has been validated in the use of chronic (over weeks or months; (Matthew S. Brook *et al.*, 2015)) and acute settings (i.e. over 2-8 days (Wilkinson *et al.*, 2014) and over 3h (Wilkinson *et al.*, 2015)). Here we present a study design with a single blood sample and a single biopsy to measure integrated MPS over 3-days.

Combination of these tracer approaches provides a minimally invasive stable isotope approach to measure MPS, MPB and muscle mass simultaneously, or at least in close proximity. Here we present and test a protocol that generates results/rates that are consistent with the literature in terms of MPS, MPB and muscle mass.

Muscle mass measured by D_3-Creatine

The use of 30 mg labelled tracer was based upon the dosing validation by Clark et al. 2014, whereby 30 mg was identified as adequate for the quantification of muscle mass. In contrast to their results, the dose spill over in the 0-24h pooled urine sample from our study was

significantly lower, and which may remove the requirement for a 24h urine collection and measurement of urinary creatine 'spillover'. However, the reasons for this are unclear, although it is assumed that the differences in percentage spillover reflects a rate of Creatine production that exceeds uptake by the muscle (Shankaran *et al.*, 2018). Furthermore, a significant correlation was only found between muscle mass measured by D_3-creatine and ALBM calculated from DXA and not with LBM by DXA (p=0.098). This may be as a result of the fact that DXA does not directly measure muscle mass, thereby supporting the findings of Proctor et al. (1999) who found a reduced relationship between urinary creatinine excretion method and DXA LBM with age. In addition, the values for DXA LBM are considerably higher (~40%) than the muscle mass measured using D_3-creatine. If DXA LBM is taken as a proxy of muscle mass, this highlights the grave and erroneous overestimation of muscle mass using DXA (Evans *et al.*, 2019). As such, ALBM by DXA has been identified as a more accurate assessment of muscle mass in humans than LBM (Heymsfield *et al.*, 1990; Dympna Gallagher *et al.*, 1997) and here showed a significant correlation with LBM (r=0.89, p=0.0011). Furthermore, Clark et al. (2014) have previously validated D_3-creatine against MRI and showed a strong correlation (r= 0.868, p<0.0001). As such, a limitation to this study was the use of DXA as the only comparison to muscle mass. The use of MRI as a gold standard would have provided a greater insight into the alignment of muscle mass measured by D_3-Creatine with a gold standard measurement and potentially less bias than with DXA. An additional

limitation is that 24h urinary creatinine concentration was not measured. However, this measure could aid assessment of the 24h urine completions.

Another point to consider with the D_3-creatine method is the use of 4.3g/kg as the value of creatine per kilogram of muscle mass. In this study, our values are similar to that of Clark et al. (2014) who also assessed older adults. However, previous studies have found that a different concentration of phosphocreatine exists between type I and type II fibres (Greenhaff *et al.*, 1994). In humans, phosphocreatine makes up around 60-70% of total creatine. With age change in muscle fibre composition has been observed, whereby a greater decrease in type II fibres with age was identified (Lexell, Taylor and Sj, 1988). Additionally, the creatine concentration within different muscle groups has been shown to vary (Mangun and Myers, 1939). Stimpson et. al. (2012) identified different enrichments of D_3-creatine in different muscles in rodents. The difference for this was presumed due to the varying fibre-type composition, their glycolytic activity and creatine uptake as described by Brault et al (2003). Therefore, future investigations need to assess whether a change in the fibre-type composition, such as with ageing, significantly alters the averaged constant of 4.3 g/kg. If the concentration was to drop, the use of the 4.3 g/kg would result in an underestimation of muscle mass. If the concentration was to be higher than 4.3 g/kg, this would result in an overestimation of muscle mass for those individuals.

Muscle protein breakdown measured by D_3-3-methyhistidine

Herein, 10 mg of D_3-3-methyhistidine was utilised, similar to that previously described by Sheffield-Moore et al. (2014). The rates of MPB in older men from our plasma samples were in agreement with the rates observed by Sheffield-Moore et al. (2014). It is important to acknowledge that the subjects were not fasted for the whole 6-hour period, therefore, the rates presented are not basal. Participants were fed half-way through the 6-hour sampling period. However, a comparison would need to be made against a completely fasted period to identify if the method was able to detect a decrease in MPB rates with feeding. However, the rates in this study were similar to the fasted rates measured by Sheffield- Moore et al. (2014). Therefore, when combining these rates with the integrated rates of myofibrillar MPS it may result in a positive contractile protein net balance. As such, the relationship between these measures should be looked into further to assess if the measures correspond with one another.

We further applied the 3MH approach to identify its sensitivity in detecting change, where a change in muscle mass may be anticipated (i.e. during immobilisation or a resistance exercise training programme), but where the mechanism of that change is unknown i.e. changes in MPS or MPB or indeed both. Clearly, it is important that we know the mechanism by which muscle mass is regulated in health and disease, so that the appropriate intervention may be targeted to increase or maintain mass or prevent loss. Currently, there is still a wide gap in understanding the role of protein turnover

not just MPB (Crossland *et al.*, 2019), partly due to the complexity of traditional methodologies used to measure MPB (Rudrappa *et al.*, 2016). In collaboration with Tessa-Morris Paterson, the 7-day bedrest study demonstrated there was suppression in whole-body MPB, using D_3-3MH. In contrast, a study completed by Symons et al. (2009) utilizing the fractional breakdown rate (FBR) approach to measure MPB, found no significant difference in the rate of MPB after 21 days of simulated microgravity (Symons *et al.*, 2009). However, in contrast, some studies have identified a suppression in muscle protein synthesis and have suggested a suppression in MPB can also occur with immobilization (Urso *et al.*, 2006; Gustafsson *et al.*, 2010). Additional measures of MPS and muscle mass in this study will aid understanding of muscle protein turnover and the significance of this MPB decrease.

We further investigated the use of D_3-3MH in a study involving 6-weeks of whole-body resistance exercise training and a double-blind placebo vs testosterone approach in older men (This data is published by Nima Gharadaghi et al. (2019)). Rates of MPB were measured at baseline, 3 and 6 weeks, although no significant differences between groups or time points was identified, a slight trend was measured in the Testosterone group at 6-weeks with RET ($p=0.11$). In this study, the rates of MPS and MPB (also assessed by estimated FBR) increased with RET in the Testosterone group. MPB is an important factor in the increase of muscle protein turnover with RET although, not to the same extent as MPS (Tipton, Hamilton and Gallagher, 2018). Importantly, net balance calculations identified that the rates of MPS

were greater than MPB. As suggested (Ferrando *et al.*, 2002), when a positive net protein balance was sustained over time, increases in muscle mass and muscle strength were identified (Gharahdaghi *et al.*, 2019). These studies therefore demonstrate the importance of assessing net balance to identify if MPS is greater than/less than MPB, in driving changes in muscle mass.

To conclude the results from this combined stable isotope tracer approach, provide rates of MPS, MPB and estimates of muscle mass that are in agreement with previous individual tracer approaches. Thus, demonstrating feasibility of this minimally invasive combined stable isotope tracer method to concurrently measure muscle mass, MPS and MPB. Further investigations and the use of this combined tracer approach in an elderly population of both men and women will be discussed in the following Chapter. In addition, the use of D_3-Creatine to measure changes in muscle mass following an exercise intervention will be presented in Chapter 5.

5. Application of the combined oral stable isotope tracer approach to assess muscle protein synthesis, muscle protein breakdown and muscle mass in a large cohort of older adults

5.1. Introduction

With the UK experiencing an ageing population (Office of National Statistics, 2019), optimising stable isotope techniques for quantifying skeletal muscle 'health biomarkers' (i.e. muscle mass, muscle protein synthesis (MPS) and breakdown (MPB)) is crucial in furthering research into the age-related loss of muscle mass and function (sarcopenia) in older adults. Moreover, any such methods must be broadly applicable, minimally invasive, robust and cost-effective if they are to be used for comprehending, and developing treatments relating to mitigating sarcopenia.

Despite differences in clinical paradigms for the identification of sarcopenia, within the literature, all definitions collectively agree that confirmation of sarcopenia requires the quantification of whole-body muscle mass. The most commonly used approaches for measurement of whole-body muscle mass are typically imaging techniques including dual-energy X-ray absorptiometry (DXA), magnetic resistance imaging (MRI), computed tomography (CT) or ultrasonography (Heymsfield *et al.*, 2015). MRI is the gold-standard for the measurement of muscle mass; however, due to its accessibility and cost, most research facilities will use DXA as the alternative. Due to the barriers presented with the use of MRI, most definitions of sarcopenia provide cut-off DXA values for the identification of sarcopenic individuals, whilst other definitions also provide bioimpedance analysis (BIA) cut-off references (Cruz-Jentoft *et al.*, 2010, 2018; International Working Group on Sarcopenia, 2011;

Studenski *et al.*, 2014). BIA is another commonly used approach for the estimation of body composition, particularly fat mass and muscle mass. However, in relation to the identification of sarcopenia, utilising imaging techniques for the quantification of muscle mass in a greater clinical population (e.g. care homes) may be limiting. D_3-Creatine is a minimally invasive technique that can be used to measure muscle mass through tracer ingestion and subsequent urine sampling (Clark *et al.*, 2014). In the recent (2018) consensus for sarcopenia by the European Working Group on Sarcopenia in Older People (EWGSOP), the use of D_3-creatine dilution technique for the measurement of muscle mass has been acknowledged as a potential method for assessment of low muscle mass, although further research and validation is required (Cruz-Jentoft *et al.*, 2018).

The advancement of stable isotope tracer techniques to study muscle protein turnover (i.e. MPS and MPB) has improved greatly. More recent uses of deuterium oxide (D_2O) for the measurement of muscle protein fractional synthetic rate as a proxy for MPS has provided the ability to assess individuals in a "free-living" environment, in a minimally invasive fashion whilst requiring a less laborious set-up. As such, this reduces cost in comparison with the more traditional approaches involving infusion of sterile isotopically labelled amino acids (Wilkinson *et al.*, 2015).

The recent reintroduction and development of D_3-3-methyhistidine (D_3-3MH) technique (Sheffield-Moore *et al.*, 2014) to assess whole-

body muscle myofibrillar protein breakdown highlights a less invasive alternative to other current stable isotope approaches in the assessment of MPB (i.e. via fractional breakdown rate or arteriovenous amino acid kinetics). With ageing, a slower loss of muscle mass is observed with women than men (Forbes and Reina, 1970; D Gallagher *et al.*, 1997). Therefore, it is important to identify if there are differences in net protein balance (i.e. where MPB>MPS) that may be driving that change. A number of studies have observed sex-differences in MPS rates (Smith *et al.*, 2008, 2009, 2012; Henderson *et al.*, 2009) but, due to the difficulty in assessment only a few have additionally assessed MPB rates (Jahn *et al.*, 1999; Fujita *et al.*, 2009). The use of D_3-3MH method to highlight any potential sex-related differences or relationships with MPS have not yet been investigated.

Consequently, the aims of this study were two-fold. First, to devise and validate the use of all three approaches to investigate the potential of a combined oral stable isotope assessment of muscle (COSIAM) approach to concurrently quantify muscle protein turnover (i.e. MPS and MPB) and muscle mass using the stable-isotope tracers D_2O, D_3-3MH and D_3-Cr, respectively. Second, was to uncover novel relationships between MPS/MPB and muscle mass in older males and females.

5.2. Methods

5.2.1. Subjects and protocol

Before enrolment onto the study, all subjects provided written and informed consent. This study conformed to the Declaration of Helsinki and received ethical approval from the University of Nottingham Faculty of Medicine and Health Sciences Research Ethics Committee (A08122015 SoMS GEM PhD).

Thirty-seven healthy older volunteers (21 males: 16 females) completed a 4-day study utilising D_3-Creatine, D_3-3-methylhistidine and D_2O simultaneously. The protocol for this study has been previously described in Chapter 2 Section 2.1

5.2.2. Dosing and Sampling

Muscle Mass determination using D_3-Creatine

Volunteers were asked to provide a single urine sample before ingesting 30 mg of D_3-Creatine (CK Isotopes) dissolved in deuterium oxide (70% APE). Following ingestion of the tracer, subjects were provided with two 3L containers for all urine collection over the next 24h. Single spot urine samples were provided at 48 and 72h after tracer ingestion. 1 ml aliquots of the urine were taken and stored in eppendorfs at -80°C until subsequent analysis. The total volume of 24h collection was recorded before an aliquot was taken (male: ~1.95L, female: ~2.3L). Urine samples were prepared for analysis on the Liquid Chromatograph- Mass Spectrometer (Q Exactive Orbitrap, Thermo Hemel Hempstead, UK) as previously described in Section 2.5.

Muscle myofibrillar protein breakdown determined using D_3-3-methylhistidine

To correct for any background *D_3-3-methylhistidine* enrichment, a single blood sample was collected in a lithium-heparinised tube before tracer ingestion. Volunteers were provided with 10 mg of D_3-3-methylhistidine (CK Isotopes) dissolved in 50 ml of H_2O to take at home 24h prior to their second visit in our labs. Volunteers returned to site for hourly blood sampling over a 6-hour period ~24h post-tracer ingestion. Plasma samples were prepared for analysis on the Liquid-Chromatograph- Mass Spectrometer (Q Exactive Orbitrap, Thermo Hemel Hempstead, UK) as previously described in section 2.7.

Deuterium Oxide

Subjects provided a baseline saliva sample before ingesting 150ml of D_2O (70% APE). 3 h post-ingestion of D_2O the volunteers provided another saliva sample to identify peak enrichment of the tracer. To observe body water enrichment throughout the study, volunteers were asked to provide further saliva samples at 12, 24, 48, and finally at 72h post-tracer ingestion. Saliva samples were prepped and analysed on the TCEA-IRMS (See Section 2.6.1 for more detail). Muscle samples were prepped and analysed on the GC-pyrolysis-IRMS (See section 2.6.2 for more detail).

5.2.3.Statistical Analysis

All data sets were tested for normality. All data are presented as mean and 95% CI, unless stated otherwise. Unpaired two-tailed t-tests were used to calculate differences between males and females. Linear regression and Pearson's product-moment correlation were used to assess relationships between muscle mass assessment methods, MPS and MPB and muscle function measures.

5.3. Results

5.3.1. Subject Characteristics

The following table provides the subject characteristics from the study. Values displayed are mean ± SD.

Abbeyfield				
		Male	Female	P value
n		21	16	-
Age (y)		72.1 ± 4.7	70.7 ± 4.2	ns
Height (m)		1.75 ± 0.087	1.62 ± 0.065	<0.0001
Weight (kg)		80.4 ± 12.7	67.6 ± 10.0	0.0044
BMI (kg/m^2)		26.1 ± 3.6	25.8 ± 3.5	ns
LBM by DXA (kg)		52.8 ± 4.3	39.7 ± 2.9	<0.0001
Total ALBM		23.8 ± 2.8	16.9 ± 1.9	<0.0001
Handgrip Strength (kg)		38.0 ± 5.2	25.8 ± 3.5	<0.0001
MVC 70 (Nm)		153.6 ± 34.5	97.6 ± 21.0	<0.0001
Single leg 1RM leg extension (kg)		36.8 ± 11.3	21.2 ± 6.2	<0.0001
Leg power via Nottingham Power Rig (W)		314.1 ± 116.1	162.5 ± 88.8	0.0002
Percentage Sarcopenic based on EWGSOP (%)	**Males**: ASMI<7.26kg/m^2, handgrip <30kg **Females**: ASMI<5.67kg/m^2 handgrip <19kg	5	0	-
Percentage Sarcopenic based on muscle mass cut-offs (%)	**EWGSOP/IWGS** **Males**: ASMI<7.26kg/m^2, **Females**: ASMI<5.67kg/m^2	24	13	-
	EWGSOP2 **Males**: ASMI<7kg/m^2 **Females**: ASMI<5.5kg/m^2	10	0	-
	FNIH **Males**: ALBM<19.75kg **Females**: ALBM<15.02kg	0	13	-

Table 5.3.1-1 Subject characteristics. P value from differences observed between males and females. Ns- denotes no significant difference between male and female (p>0.05).

5.3.1.Muscle mass using D_3-Creatine

5.3.1.1. *Labelled Creatine in Urine*

The greatest ratio of D_3-creatine to unlabelled creatine was during the 24 h urine pool and that this then decreased significantly for further sampling for both males and females Figure 5.3.1.1.

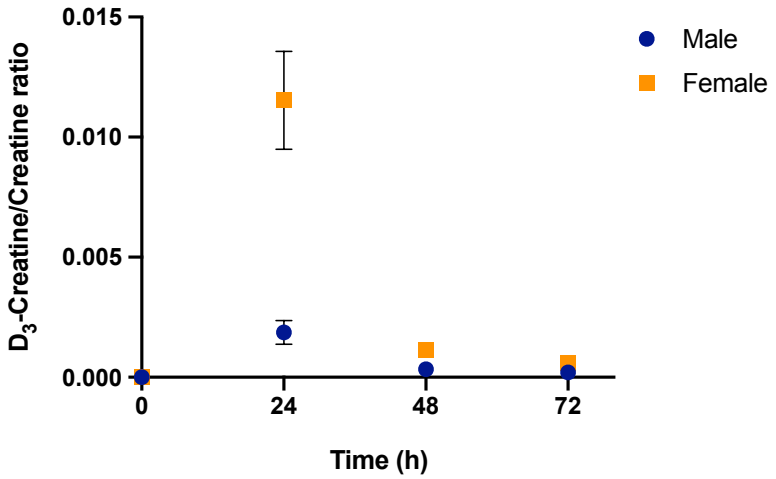


Figure 5.3.1.1 Corrected ratio of D_3-Creatine/Creatine excreted in the urine for samples collected at Baseline (0h), 24h pool, 48 and 72h

Using the 24h urine pooled sample, the percentage spill over could be calculated. Refer to chapter 4 for more detail. The percentage of the dose that was excreted in the urine in the first 24 h, was significantly higher in the females (5.09%; 95% CI: 1.95 to 8.23%) than males (0.25%; 95% CI: 0.046 to 0.46%), where $p < 0.0001$ (Mann-Whitney test). (See Figure 5.3.1.2).

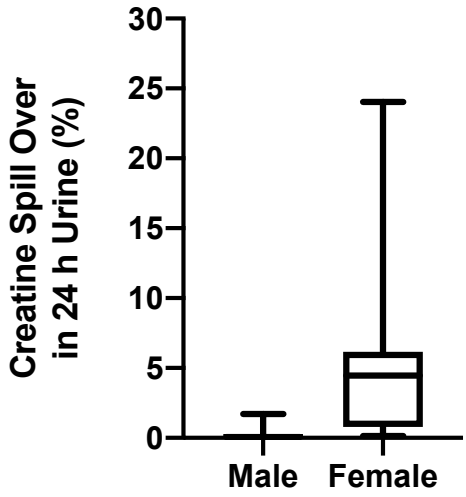


Figure 5.3.1.2. Percentage dose not absorbed by the body in 0-24 h urine collection sample. Values displayed are min to max plots.

5.3.1.2. *D_3-Creatinine Enrichment in urine over time*

The ratio of labelled D_3-Creatinine to unlabelled creatinine rose to a plateau/peak at 48h and maintaining a constant enrichment up to 72h i.e. there was no significant difference between the enrichment for the 48h and 72 h urine samples, in either the males or females. However, there were differences between males and females in the dilution of the total creatine pool.

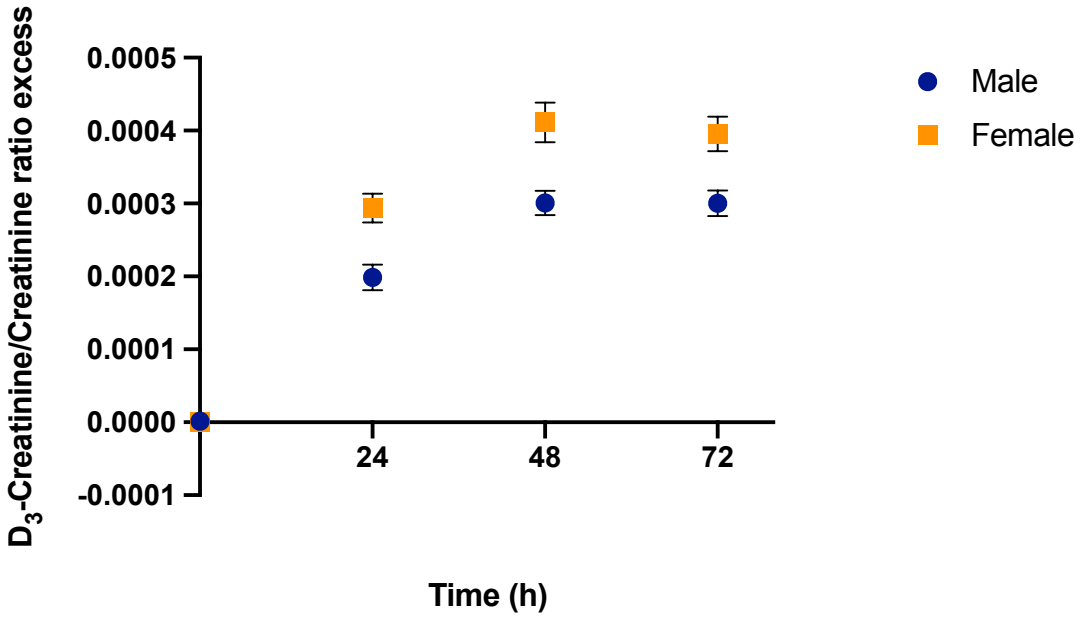


Figure 5.3.1.3 Ratio of labelled creatinine (D_3-creatinine) to unlabelled creatinine, corrected to the baseline sample (0h) for 24,48 and 72h urine samples in both males and females. Mean ± 95% CI.

5.3.2.Muscle architecture

There was no significant difference between males and females in thickness of the m. vastus lateralis (VL), in fascicle length or pennation angle.

	Male	Female
VL thickness (cm)	2.08 ± 0.38	1.88 ± 0.31
Fascicle length (cm)	8.46 ± 0.94	8.03 ± 1.22
Pennation angle (˚)	14.98 ± 3.04	14.44 ± 1.95

Table 5.3.2-1 Muscle architecture measurements. Values displayed as mean ± SD.

5.3.3.Myofibrillar fractional synthetic rate

5.3.3.1. *Body water deuterium enrichment*

A fixed volume of 150ml D_2O was ingested. The body water enrichment of deuterium was calculated in APE from the saliva

samples taken at baseline and 2, 12, 24, 48 and 72 h post D_2O ingestion. Females had a significantly ($p<0.05$) higher level of enrichment than the males at each time point (See Figure 5.3.3.1A). The mean deuterium enrichment over the 72h period for males and females was 0.21 (95% CI: 0.25 to 0.29 APE) and 0.27 APE (95% CI: 0.20 to 0.23 APE), respectively (See Figure 5.3.3.1B).

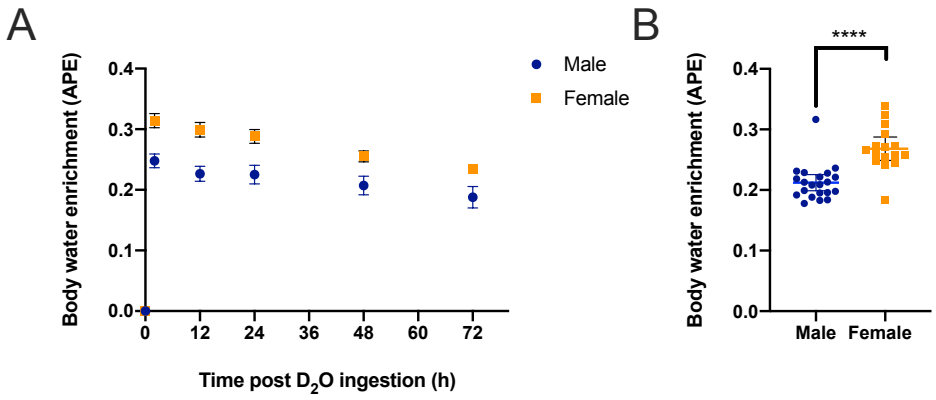


*Figure 5.3.3.1 A) Deuterium enrichment of the body water, measured in APE from saliva samples at baseline, 2, 12, 24, 48 and 72h post- deuterium oxide ingestion. B) Mean deuterium enrichment over the 72h time period in males and females. **** denotes p<0.0001*

5.3.3.2. *Myofibrillar fractional synthetic rates*

There was no significant difference between the male (0.064 %/h, 95% CI: 0.057 to 0.07 %/h) and female (0.071%/h, 95% CI: 0.065 to 0.078 %/h) myofibrillar fractional synthetic rates from the Abbeyfield study, but a strong trend where p= 0.083 (two-tailed unpaired t-test). Figure 5.3.3.2.

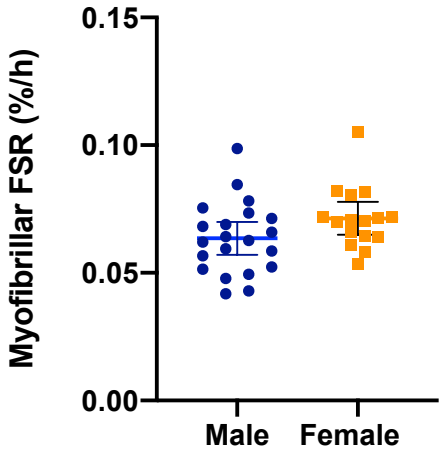


Figure 5.3.3.2 Myofibrillar fractional synthetic rates for males and females from the Abbeyfield study. Mean and 95% CI.

5.3.4.Whole-body muscle protein breakdown rate using D_3-3MH

5.3.4.1. *Dilution of D_3-3-methylhistidine*

The enrichment of D_3-3-methylhistidine in the plasma samples was significantly higher in the females than the males of the Abbeyfield study. This is due to a single bolus being provided and not per body weight. Figure 5.3.4.1 displays the decay curves where the area under the curve (AUC) was significantly higher for the females (34.4 %.h, 19.1 to 39.7 %.h) than the males (27.1%.h, 23.1 to 31.1%.h) (p=0.024 calculated using a two-tailed unpaired t-test).

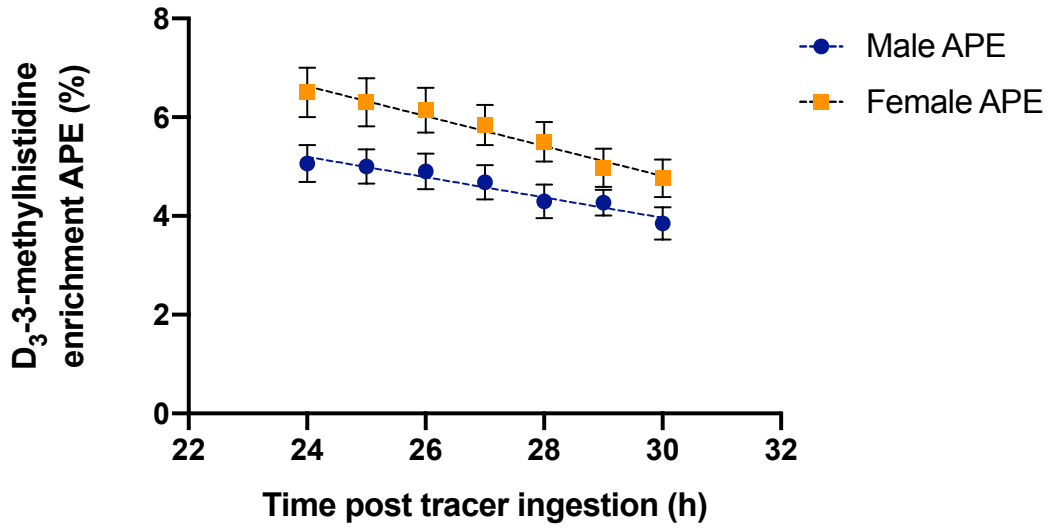


Figure 5.3.4.1 Decay curves of D_3-3-methylhistidine in the plasma by endogenous 3-methylhistidine release for both males (n=21) and females (n=16). Mean and 95% CI.

5.3.4.2. *Rates of whole-body muscle protein breakdown*

There was no significant difference between the rates of whole-body muscle protein breakdown, defined with the rate constant, k, between males (0.057; 0.046 to 0.068 k) and females (0.054; 0.037 to 0.070 k). (Figure 5.3.4.2).

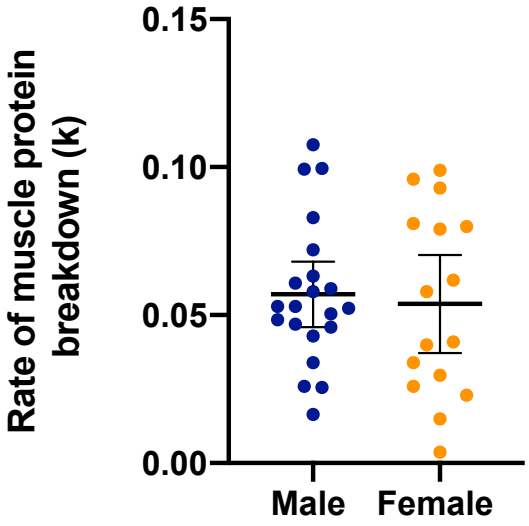


Figure 5.3.4.2 Rate of whole-body muscle protein breakdown (k) using D_3-3-methylhistidine in males (n=21) and females (n=16). Mean and 95% CI.

5.3.5.Comparison of D_3-Creatine method vs DXA

There is a significant positive correlation between muscle mass measured by the D_3-Cr method and both lean body mass (LBM) and appendicular lean body mass (ALBM) by DXA in both males and females. When male and female data are combined, there is a strong significant positive correlation between muscle mass measured by D_3-Cr and LBM and ALBM measured by DXA (See Figure 5.3.5.1). There was a significant difference between muscle mass by D_3-Cr and ALBM by DXA ($p=0.048$, using a one-way ANOVA with repeated measures and Tukey's *post hoc* test). LBM by DXA was significantly higher than both ALBM and muscle mass by D_3-Cr, with values for LBM approximately 40% higher.

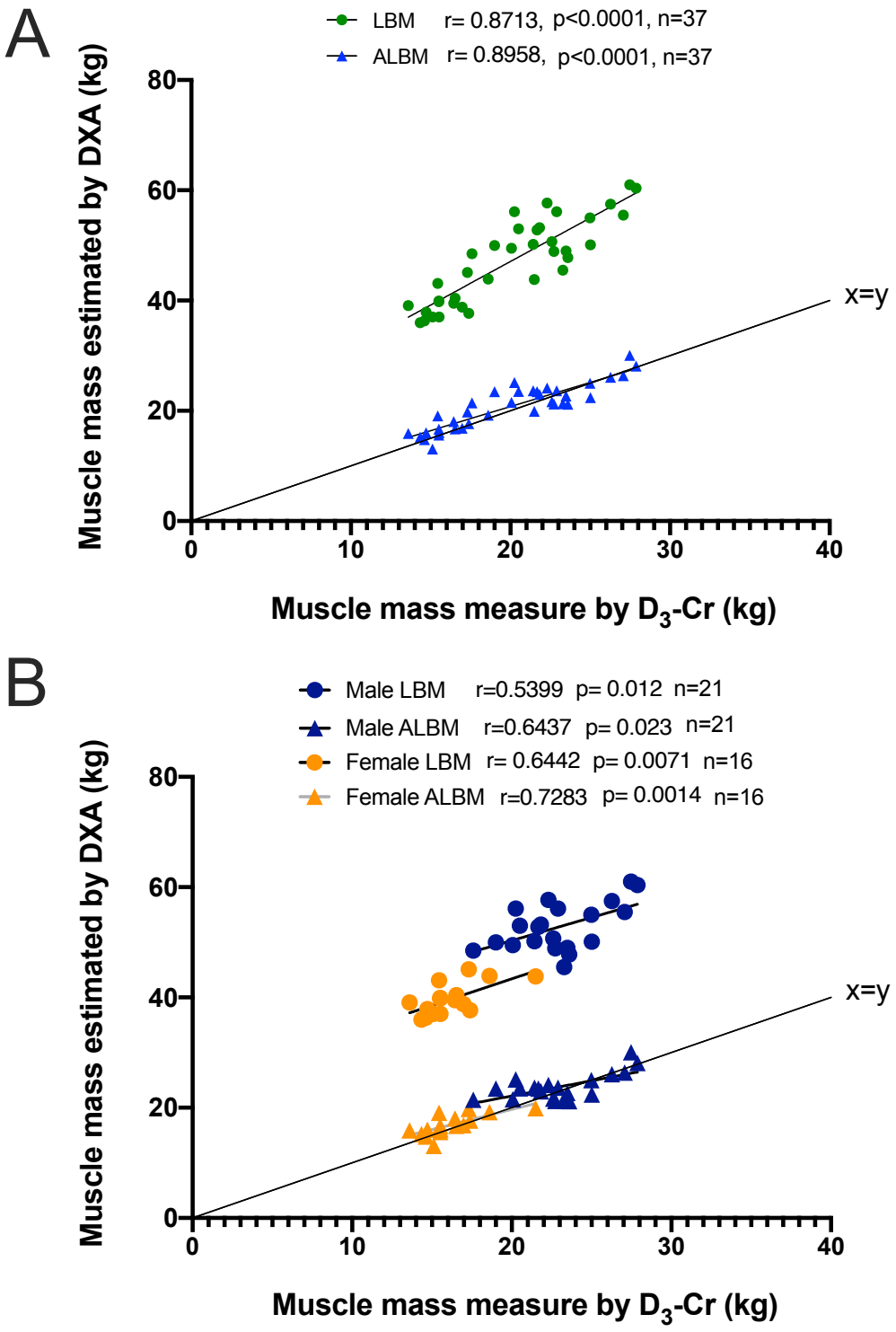


Figure 5.3.5.1 A correlation between muscle mass measured by D_3-Creatine method with lean body mass (LBM) measured by DXA and appendicular lean body mass (ALBM) when data are combined (A) and separated by gender (B).

5.3.1. Correlation between muscle mass measured by D3-Creatine and muscle thickness by ultrasound

When data for males and females was combined there was a significant correlation between whole-body muscle mass measured by D_3-creatine and m. vastus lateralis thickness measured by ultrasound (See Figure 5.3.1.1B). When combined, there was a significant positive correlation between DXA LBM and VL thickness (r=0.375, p=0.026, n=35). However, when the data was separated by gender, there were no significant correlations between the measures by DXA or D_3-Cr (See Figure 5.3.1.2).

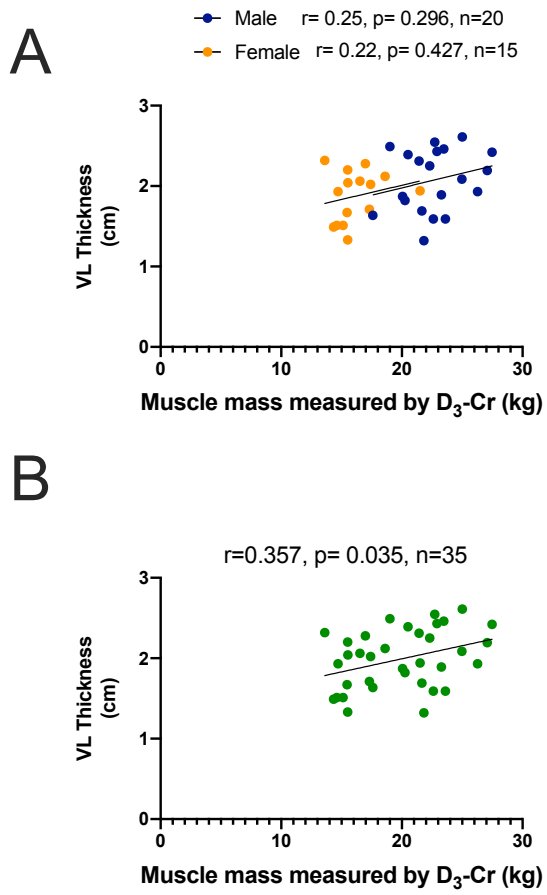


Figure 5.3.1.1 Correlation between muscle mass measured by D_3-Creatine and m. vastus lateralis thickness measured by ultrasound separated by male and female (A) and combined (B).

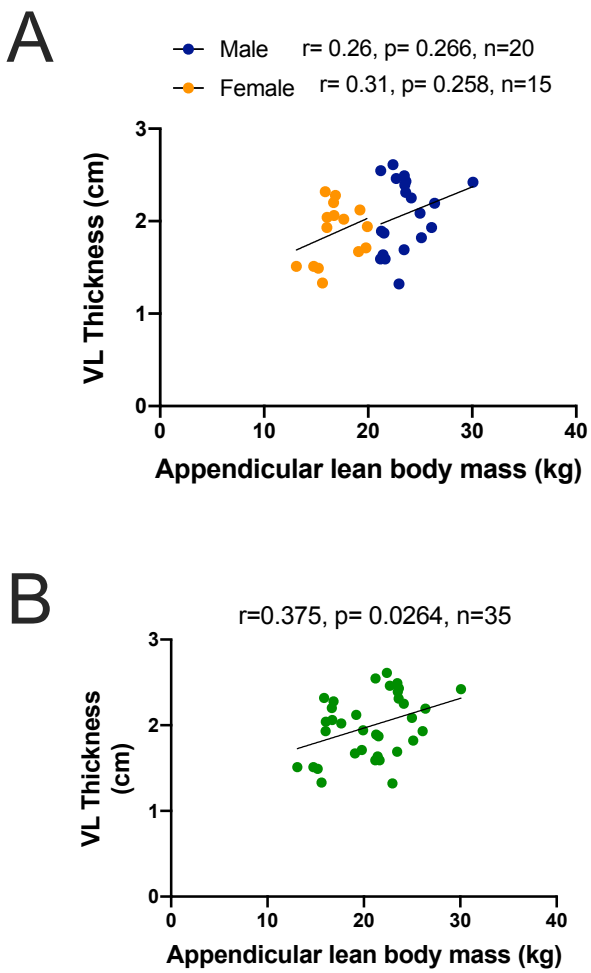


Figure 5.3.1.2 Correlation between appendicular lean body mass and m. vastus lateralis thickness measured by ultrasound separated by male and female (A) and combined (B).

5.3.2.Relationships between muscle mass and muscle strength and power

When all data (i.e. male and female) are combined, there are significant correlations ($p<0.0001$) for muscle mass measured by D_3-Creatine and single-1RM leg extension, handgrip strength, leg power and single leg MVC (See Figure 5.3.2.1). This is due to the female cohort having lower muscle mass and lower muscle strength than the males, therefore, when combined a positive correlation is evident. Therefore, data was separated by gender to identify any gender-specific correlations between muscle mass and muscle strength

measures (See Figure 5.3.2.2). There was no significant correlation between 1-RM and muscle mass measured using D_3-Creatine (females: r=0.44, p=0.13) although there was a slight trend for the males (r=0.44, p=0.067). There was a no significant correlation between muscle mass measured by D_3-Cr and MVC in the males or females. Although a trend was identified in the females (p=0.074). In addition to MVC and 1RM leg extension, the Abbeyfield study assessed handgrip strength, SPPBT and leg power via the Nottingham power rig. A significant correlation was only found between leg power and muscle mass measured by D_3-Creatine in the males (r=0.44, p=0.049).

Appendicular lean body mass also had significant positive correlations with single-1RM leg extension, handgrip strength, leg power and single leg MVC, when male and female data was combined (See Figure 5.3.2.3). When data was separated by gender, there was no significant correlations for male or females between ALBM and single leg 1RM extension or leg power. However, there was a significant positive correlation between handgrip strength and ALBM only for the females and not males (p=0.0197). There was also a strong trend between ALBM and single-leg MVC for the females and not the males (p=0.0533). (See Figure 5.3.2.4)

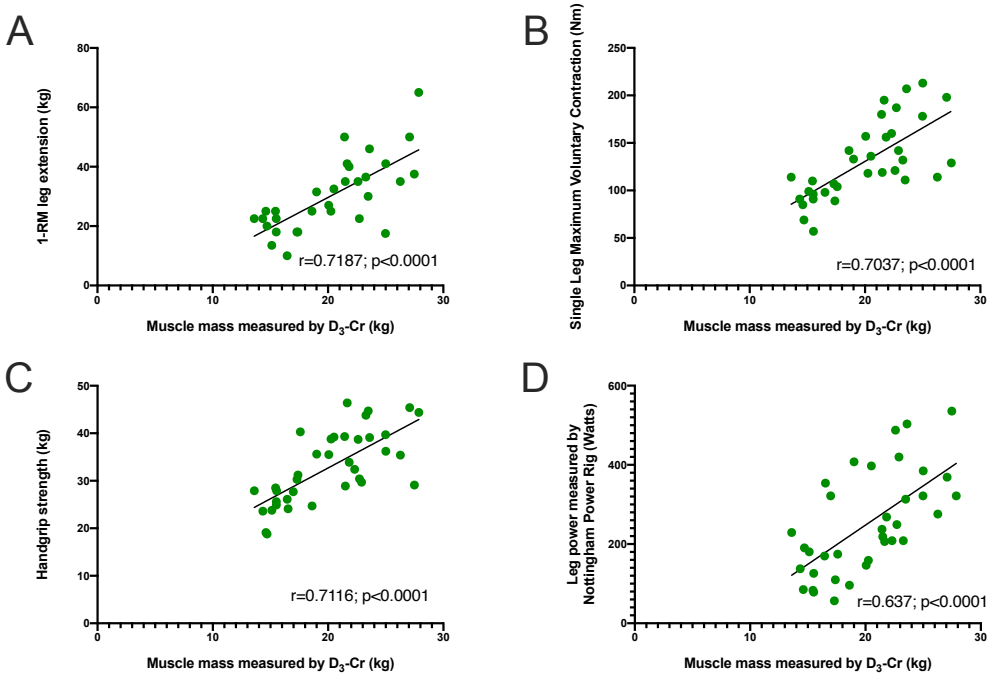


Figure 5.3.2.1 Correlations between muscle mass measured by D_3-Creatine with (A) single-leg 1RM leg extension, (B) single leg maximum voluntary contraction, (C) handgrip strength and (D) leg power for both men and women combined.

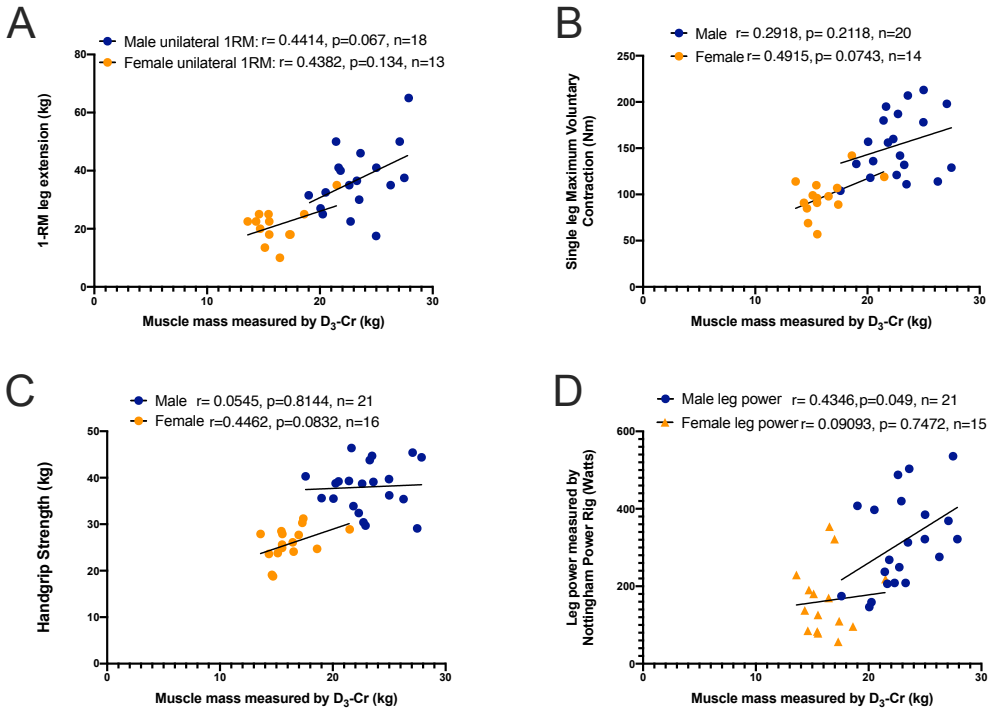


Figure 5.3.2.2 Correlations between muscle mass measured by D_3-Creatine with (A) single-leg 1RM leg extension, (B) single leg maximum voluntary contraction, (C) handgrip strength and (D) leg power for both men and women.

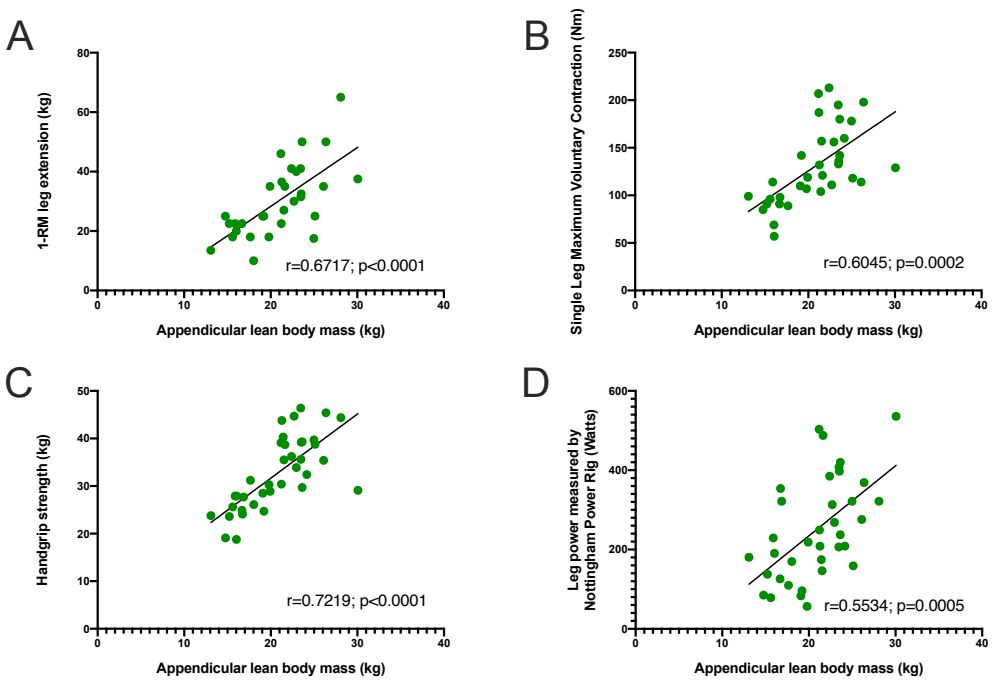


Figure 5.3.2.3 Correlations between appendicular lean body mass with (A) single-leg 1RM leg extension, (B) single leg maximum voluntary contraction, (C) handgrip strength and (D) leg power for both men and women combined.

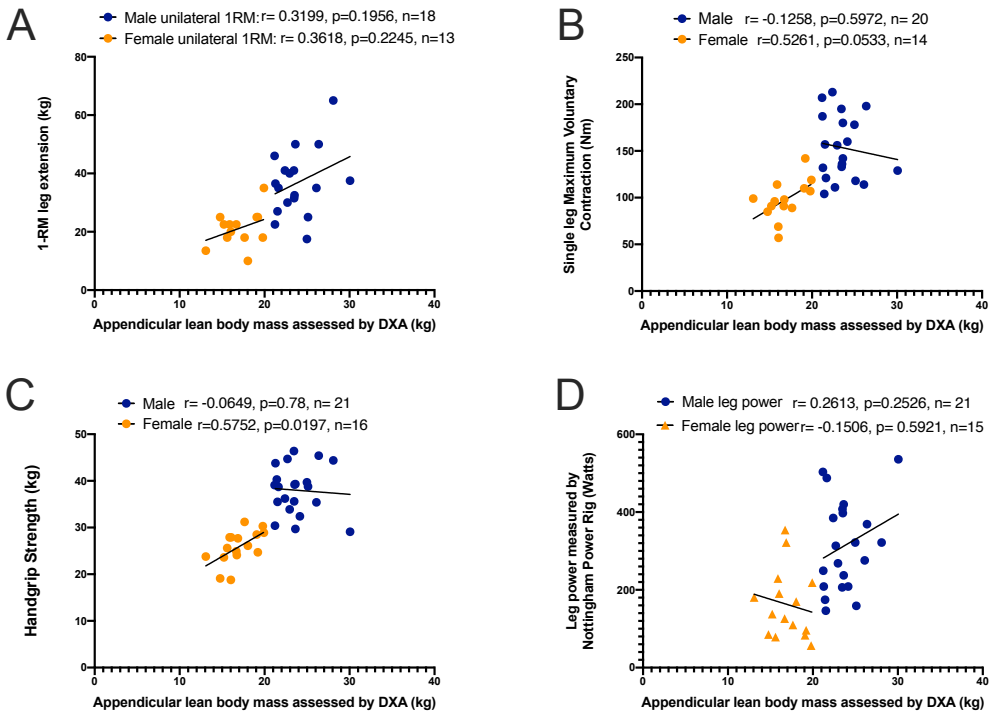


Figure 5.3.2.4 Correlations between appendicular lean body mass with (A) single-leg 1RM leg extension, (B) single leg maximum voluntary contraction, (C) handgrip strength and (D) leg power for both men and women.

5.3.3. Identifying relationships between muscle mass and muscle protein turnover (MPS & MPB)

There was no significant correlation between LBM or ALBM and FSR (see Figure 5.3.3.1) when data was combined or separated by gender. Furthermore, there was no significant correlation between FSR and muscle mass measured by D_3-Creatine. There was no significant correlation between muscle mass measured by DXA or by D_3-Cr and whole-body MPB when data was combined or separated by gender (See Figure 5.3.3.2). Net balance (i.e. MPS-MPB) was calculated for each individual to identify if they were in a positive or negative state of protein turnover and whether this instead may correlate with muscle mass. When combined or separated by gender there was no significant difference between net balance (%/h) and muscle mass measured by DXA or D_3-Creatine (See Figure 5.3.3.3)

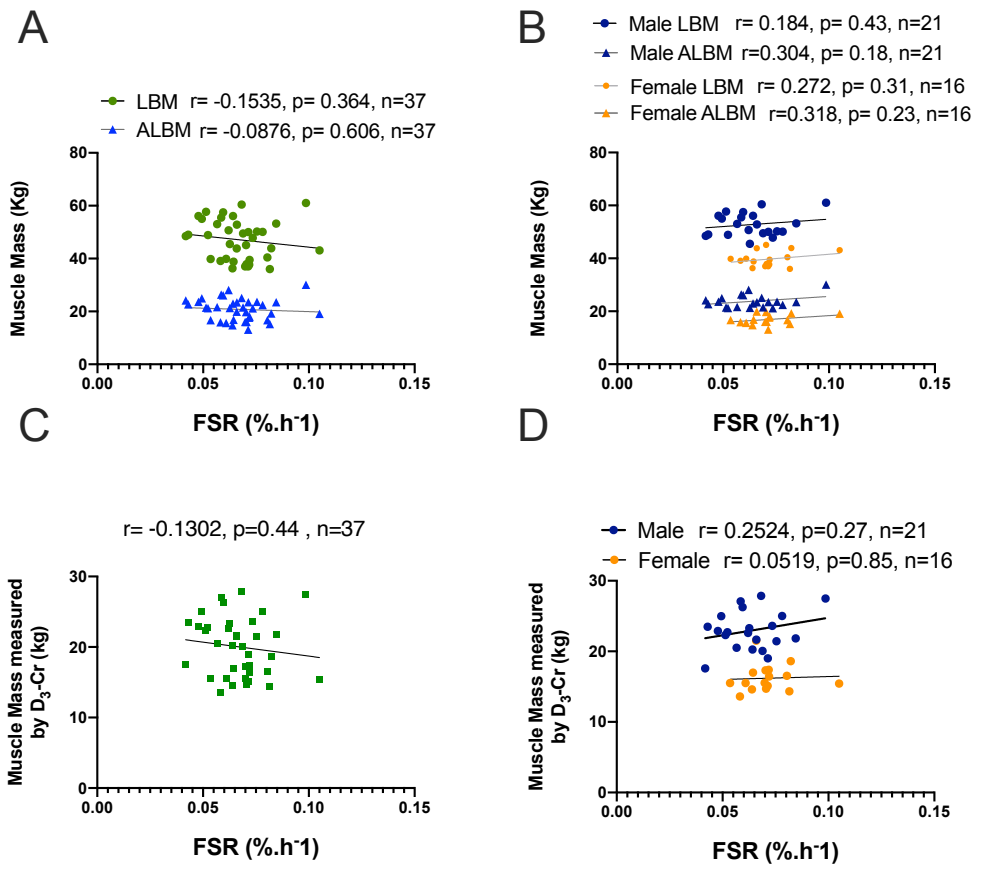
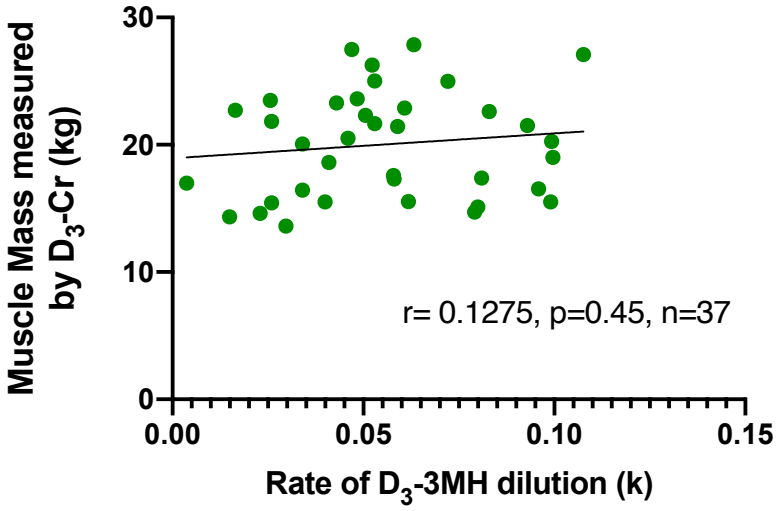


Figure 5.3.3.1 Linear regression of DXA lean body mass (LBM), appendicular lean body mass (ALBM) measures with fractional synthetic rate (FSR) of the m. vastus lateralis where data are combined in A) and separated by gender in B). Linear regression between FSR and muscle mass measured using the D3-creatine method with data combined (C) and separated by gender (D).

A



B

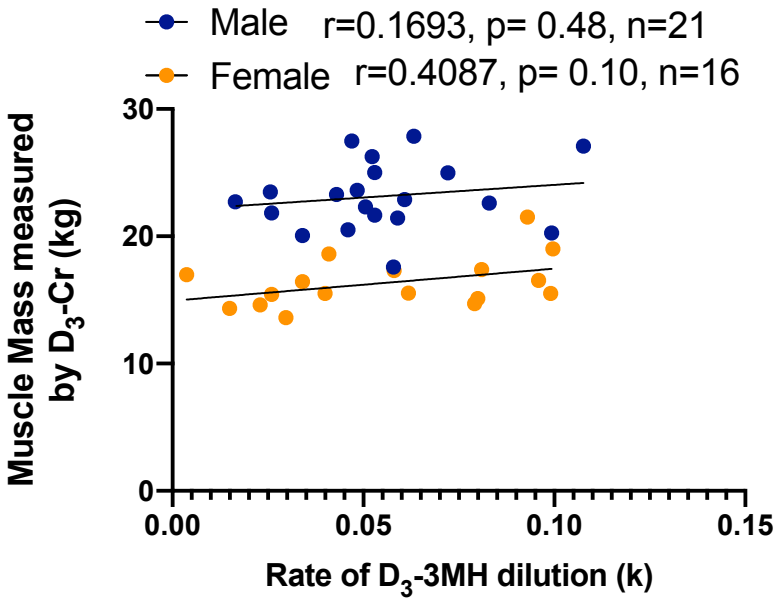


Figure 5.3.3.2 Linear regression of muscle mass measured by D_3-Cr and whole-body MPB with data combined (A) and separated by gender (B)

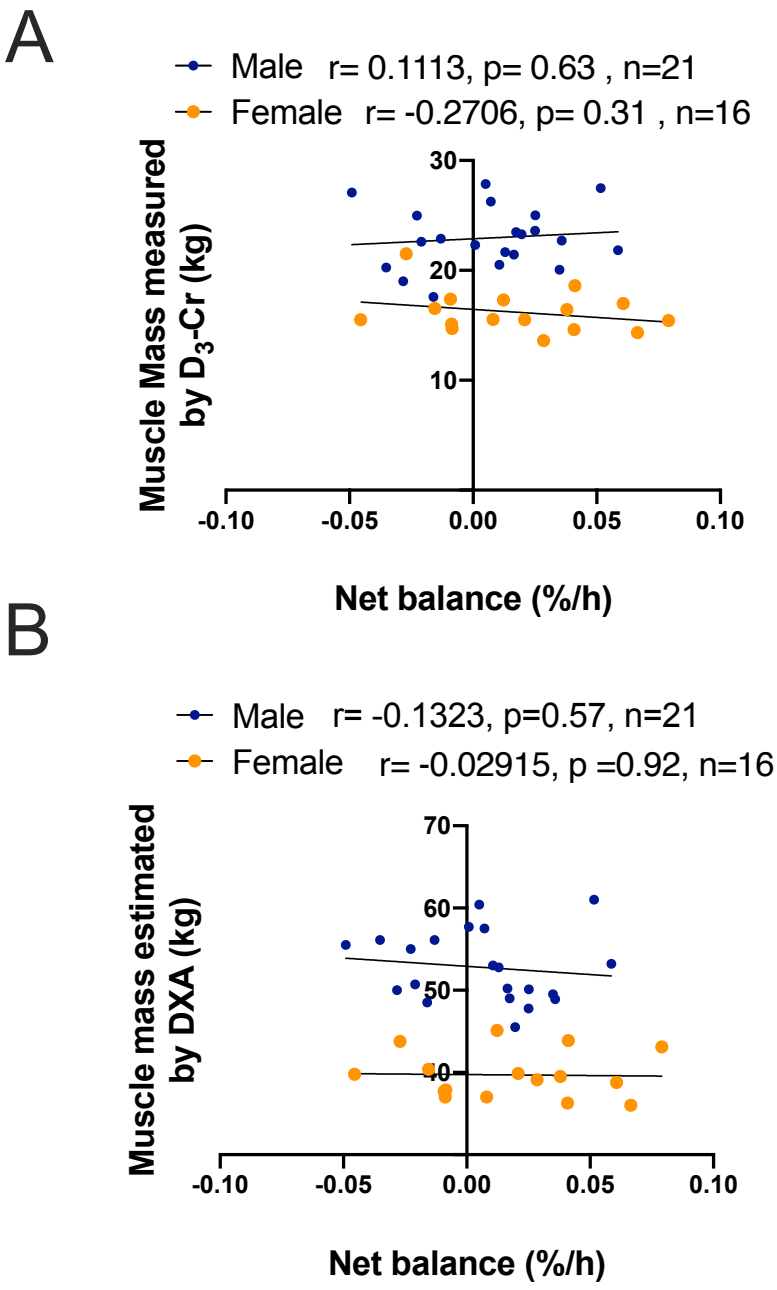


Figure 5.3.3.3 Correlations between net balance and muscle mass measured by D_3-Creatine (A) and DXA (B)

5.3.4.Relationship between MPS and MPB and MPS/MPB with muscle strength

There was no significant relationship between myofibrillar FSR of the m. vastus lateralis and whole-body muscle myofibrillar protein breakdown. (Figure 5.3.4.1). There were no significant correlations between any of the muscle strength/ function assessments and whole-body MPB or FSR.

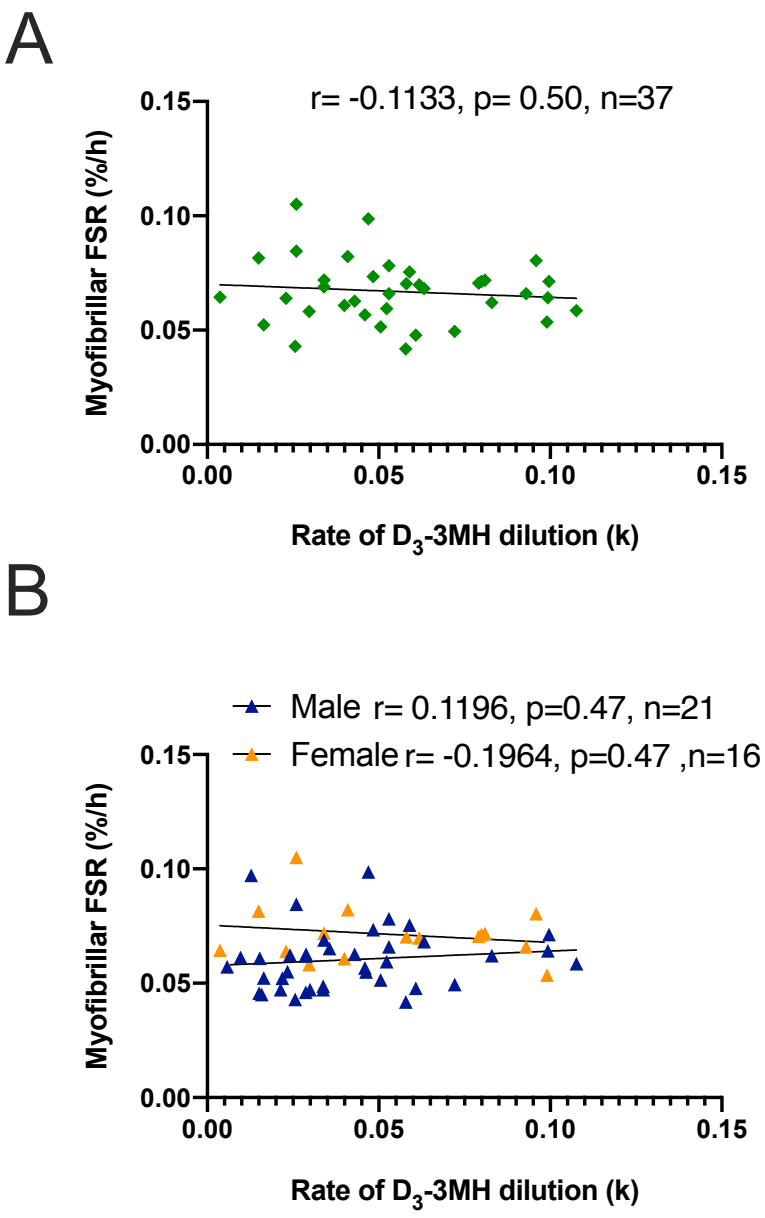


Figure 5.3.4.1 No significant correlation between myofibrillar FSR and rate of MPB represented by the rate of D_3-3MH dilution

5.4. Discussion

There has been a recent re-emergence of stable isotope techniques such as the use of D_3-3-methylhistidine for the measurement of whole-body muscle protein breakdown, D_3-Creatine for the quantification of whole-body muscle mass and the D_2O tracer technique for assessment of muscle protein synthesis. These have provided the opportunity for the development of a more minimally invasive combined approach to assess muscle protein turnover and muscle mass simultaneously in older populations. Here we have demonstrated a novel approach in which all three measures can be done concurrently in a minimally invasive fashion in older adults.

The measurement of muscle mass using D_3-Creatine

The use of D_3-Creatine to measure muscle mass is a re-emerging technique due to its close correlation with MRI measures of muscle mass (Clark *et al.*, 2014). Herein, we further support the findings of Clark et al. (2014) of the correlation of D_3-creatine method for the quantification of muscle mass against LBM measures of DXA and confirming that DXA overestimates muscle mass. This is because lean body mass (as described in the DXA measurement) is not a direct measure of muscle mass as the measure is a combination of water weight, organ weight and muscle mass (Evans *et al.*, 2019). ALBM measured by DXA provides a closer approximation of muscle mass to the D_3-creatine method, although the total LBM of the limbs is not an accurate measure of the whole-body muscle mass. Furthermore, a

study by Proctor et al. (1999) identified a reduced relationship between DXA LBM and a 24h urinary creatinine excretion method for the measurement of muscle mass. The authors suggested that this change with age may be due to an increased body water pool with age interfering with the DXA measurement (Proctor *et al.*, 1999). Despite DXA not being an accurate measure of muscle mass, it is still used in the determination of low muscle mass and identification of sarcopenic. Therefore, numerous studies that have utilised DXA in identifying sarcopenic individuals, may be underestimating the number of individuals with low muscle mass.

Approximately 98% of creatine can be found in the skeletal muscle pool, a large fraction of the remainder is carried in the brain (Hunter, 1922). Therefore, after ingestion of labelled creatine, the labelled creatine will be distributed amongst the endogenous creatine pool, of which 98% is in muscle. With the irreversible conversion of creatine to creatinine in the kidney before excretion in urine, the labelled creatine tracer will be able to provide a close assessment of muscle mass. The main advantage of the use of this minimally invasive method is that it provides a more direct estimate of muscle mass. A more accurate quantification of muscle mass than DXA (as DXA does not measure muscle mass but LBM). ALBM has been shown to correlate strongly with other estimates of muscle mass (Heymsfield *et al.*, 1990). Here, both ALBM and muscle mass measured using D_3-creatine had similar significant associations with VL thickness, when male and female data were combined. Furthermore, when male and female data was combined D_3-creatine showed slightly greater

correlations with muscle strength than ALBM. This suggests that the D_3-creatine method may provide better estimates of muscle mass than those derived from DXA. Currently, there is limited literature on the associations between muscle mass measured by D_3-creatine and muscle strength. However, a recent study by Cawthon et al. (2019) had also identified stronger associations between D_3-creatine muscle mass measures and physical performance than DXA lean mass. However, the only strength test carried out that matched this study was grip strength (Cawthon *et al.*, 2019). Therefore, future studies should further investigate the relationships between muscle mass measured using the D_3-creatine method and muscle strength and function tests.

Here, we observed clear sex-differences in the use of this method in that there was significantly higher excretion of D_3-Creatine in the urine within the first 24 h in women, than in men. This is supported by a recent finding by Clark et al. (2018) and in their previous work (2014) that found a greater excretion of D_3-creatine in women than men. The reasoning for this difference is not yet fully understood, although it is assumed that this reflects a rate of creatine production that exceeds uptake by the muscle (Shankaran *et al.*, 2018). Therefore, the potential for this method being done with a single urine sample before and after (e.g. at 48 or 72h) tracer ingestion and without the 24 h pooled sample is limited. If the spill-over is not accounted for, this can overestimate muscle mass. A 2% dose spill over (i.e. 0.6mg of 30mg) can account for ~0.5kg increase in muscle

mass if omitted. As a result, the 24h pool sample should be used to improve accuracy of the measure.

Factors that may affect the measurement of muscle mass by D_3-Creatine

Although diet was not controlled in this study, participants were asked at screening if they were taking any creatine supplements or if they were vegetarian, with one reporting as vegan. Clark et al. (2014) stated that creatine supplementation can increase the creatine pool size and therefore using the constant of 4.3g/kg can overestimate those individuals muscle mass by ~15% and that the opposite is likely to occur with individuals who consume creatine less frequently. As such, for vegetarians and vegans the calculation would underestimate their muscle mass by 15%. This is as a result of different creatine pool sizes. Creatine supplementation has been shown to increase the muscle creatine pool (from 126.8 to 148.6 mmol/kg dm after 20g/day supplementation for 7 days; (Harris, Söderlund and Hultman, 1992)), conversely in people who don't eat animal protein the muscle creatine pool is known to be smaller (reduced from 153·5 to 128·8 mmol/kg dw; (Blancquaert *et al.*, 2018)). Furthermore, aspects such as age, gender or chronic diseases should be considered. A greater decrease in type II fibres with age has been previously identified (Lexell, Taylor and Sj, 1988). Therefore, whether this change in fibre-type composition in ageing affects the creatine content needs to be considered. The total creatine content in skeletal muscle was found to differ based on the glycolytic activity of the muscle and fibre type

composition, whereby type 2 fibres expressed a higher content than type 1 (Edstrom *et al.*, 1982; Brault, Abraham and Terjung, 2003). Furthermore, the resting phosphocreatine content has been found to differ between fibre types in rats, where type 2a and b fibres had a content of ~32mM PCr and type 1 ~16mM PCr (Kushmerick, Moerland and Wiseman, 2006). Stimpson et al. (2012) identified different D_3-enrichments in different muscle types, which was presumed due to the varying fibre-type composition, their glycolytic activity and creatine uptake as described by Brault et al (2003). The 4.3g/kg coefficient was based on a study by Kreisberg et al. (1970). Although this was an estimate and not directly measured in muscle, Clark et al. (2014) identified that the value corresponded with other estimates of creatine content in muscle. Other studies have found that creatine content remains relatively stable based on age and gender (Möller and Brandt, 1982; Balsom, Söderlund and Ekblom, 1994; Sant'Ana Pereira *et al.*, 1996). However, this may need further investigation. With regards to chronic diseases, more research needs to be done within different populations to assess if there are changes in creatine concentration. Some chronic diseases have shown no change (Willer *et al.*, 2000; Weber *et al.*, 2009), however, a decrease is observed in diseases of muscular dystrophy (Fitch and Sinton, 1964). Therefore, using a fixed value of 4.3 g/kg may not be wholly representative in all populations as it may over/underestimate their muscle mass.

In this cohort, only one male was identified as sarcopenic using the EWGSOP definition for strength and function (See Table 5.3.1-1). Based on muscle mass cut-offs alone, the percentage of sarcopenic

individuals within the cohort varied from 0-24% in the males and 0-13% in the females. Therefore, with this reduced muscle mass, these subjects could potentially have an altered fibre type composition and as such, a reduced creatine concentration in the muscles. Consequently, it is possible that for these individuals their muscle mass measured using D_3-creatine could be underestimated. However, the discrepancies in the different sarcopenia muscle mass cut-offs make this difficult to determine as some of the definitions did not identify any sarcopenic participants within this cohort.

Measurement of FSR using D_2O

Due to the already well-established minimally invasive nature of deuterium oxide as a stable isotope tracer for the assessment of MPS, this study used D_2O to assess the rates of the volunteers in a "free-living" environment. A single bolus of 150 ml has previously been shown as sufficient to measure MPS over 2-8-days (Wilkinson *et al.*, 2014). As a result of a standard fixed bolus, the enrichment level of D_2O in the body water was significantly higher in the females than the males. This is due to the lower body weight of the female volunteers and also as a consequence of women having a lower percentage of total body water to weight than men and greater fat mass (48% vs 58%; (Chumlea *et al.*, 1999)). However, as enrichment is measured for each individual, this difference will not impact on the measurement of MPS in these populations.

Numerous studies have found no significant sex-differences in basal FSR rates in the young or middle-aged (Jahn *et al.*, 1999; Fujita *et*

al., 2007; Smith *et al.*, 2009), although, a small number of studies have identified differences in FSR in elderly men and women (Smith *et al.*, 2008; Henderson *et al.*, 2009). Here, the male cumulative myofibrillar MPS rates over 0-3 days (1.52 ± 0.07%/d; mean ± SEM) were similar to the rested leg myofibrillar MPS rates over 0-4 days (1.47 ± 0.06%/d) in the study by Wilkinson et al. (2014). The female myofibrillar MPS rates over 0-3 days were slightly higher (1.71 ± 0.07%/d). In contrast to those studies showing a gender bias, here we found no significant difference in cumulative myofibrillar MPS rates in older male and females, although there was a slight trend to a higher MPS rate in women (p= 0.083). However, the aforementioned studies (Smith *et al.*, 2008; Henderson *et al.*, 2009) measured basal MPS and fed MPS rather than a cumulative rate over 3-days, which combines basal and fed rates. In addition, our population may differ from those studies, as the women from this cohort seem to have a lower muscle mass and BMI in comparison with the women from the study by Smith et al. (2008). Furthermore, diet was not controlled in this study which could increase intersubject variability in comparison with controlled diet trials, where protein, carbohydrate and fat intake are controlled.

Measurement of whole-body MPB using D_3-3MH

The provision of 10mg of D_3-3MH was based on the method described by Sheffield-Moore et al. (2014). Similar to the provision of D_2O, all participants received the same amount of tracer 10mg. As such the women in this study had a higher plasma enrichment of D_3-3MH than

the men, simply due to differences in body size and muscle content. As it has been recently re-developed, there are limited studies that have used this 3MH method for a comparison. However, the rates obtained in this study are identical with Sheffield-Moore *et al.* (2014). We found no significant difference between male and female rates of breakdown. Smith et al. (2008) found no significant difference between men and women in whole-body MPB (calculated from the rate of leucine appearance in plasma), despite a ~30% greater basal mixed MPS rate in women than men. Additionally, in a study using basal amino acid kinetics using the AV balance approach, (when corrected per unit of lean mass), no significant differences in muscle protein breakdown were observed (Fujita et al. (2007)). Therefore, the results from this study are in concordance with the limited previous studies assessing MPB differences in men and women.

Factors that may affect the measurement of MPB using D_3-3MH

Diet was not controlled or monitored in this study as Sheffield-Moore et al. (2014) had found no impact of a meat diet on the dilution (decay rate) of 3MH tracer. Previous work (Huszar *et al.*, 1983) had suggested that meat protein may be a significant source of unlabelled 3MH, following protein digestion, that may influence the measurements. Since we are making acute measures of 3MH enrichment (i.e. over 6h), it is not clear if acute changes in MPB would be accurately reflected in the decay rates. Therefore, it is possible that as the volunteers were not fasted throughout the time of collection, this may affect the rate. MPB rates are suppressed in

response to insulin (Abdulla *et al.*, 2016), therefore, the dilution of D_3-3MH by endogenous 3MH may have been reduced in the latter blood sample collections. As such, the slope to determine MPB rates would be lowered, resulting in a lower k value. Although, whether the method is sensitive enough to pick up an acute feeding response is yet to be validated. However, for consistency, a light meal of a sandwich, crisps or yoghurt and drink was provided at the midpoint of the blood collections for all participants, primarily to avoid an ~18h fast. Whether the method is robustly sensitive enough to pick up acute changes in muscle protein breakdown is still yet to be fully elucidated.

Overall, we have shown that with combining these three methods in a minimally invasive approach, we can obtain concurrent measures of muscle mass, MPS and MPB. The results from each tracer measure has shown results similar to existing literature. The use of D_2O over 2-8 days has already been validated (Wilkinson et al. 2014). Our male FSR rates are in concordance with the rates from that study. Although the female rates were slightly higher ($p=0.083$), such sex-differences have been previously identified. MPB rates were also similar with previous literature. However, Further investigations and validation against another measure of MPB would be beneficial. Muscle mass estimated by D_3-creatine reported similar associations with DXA as Clark et al (2014). Additionally, similar overestimations in total lean mass by DXA were observed. Although, the relationship between D_3-creatine and MRI have been previously established (Clark et al

(2014), the use of MRI here would have aided validation of the method, by comparison with a gold-standard measure.

Relationships between muscle mass, muscle protein turnover and function

A second aim of this study was to uncover any relationships between mass, muscle protein turnover (MPS & MPB), and also muscle function using this combined three tracer approach. Muscle mass is regulated by both MPS and MPB. When both MPB and MPS are in dynamic equilibrium, muscle mass is maintained. When MPS>MPB an increase in muscle mass is observed, whilst when MPB>MPS and decrease in mass is observed. Traditional approaches to measure MPS or MPB using stable isotopically labelled amino acid tracers, would require a complex clinical set-up, involving the use of intravenous tracer infusions, multiple cannulations as well as multiple biopsies (Wilkinson *et al.*, 2015). Consequently, such studies are restricted to ~12h period, whilst requiring the participant to be bed-bound. A set-up as such may not be applicable to other populations (e.g. frail elderly/ clinical or even children). The combination of these tracer approaches has not been previously investigated.

There are clear sex-differences in the rate of loss of muscle mass with age, whereby women lose lean body mass at a slower rate than men (Forbes and Reina, 1970; D Gallagher *et al.*, 1997). Therefore, identifying if there are differences in net protein balance that may be driving these changes is important. As previously stated, a small

number of studies have observed differences in basal FSR between men and women in young (Fujita *et al.*, 2007), middle-aged (Smith *et al.*, 2009) and older individuals (Smith *et al.*, 2008); however, few studies assessed MPB. A study by Volpi et al. (2001) looked at basal amino acid kinetics to observe any differences in basal MPS or MPB across the limb (Volpi *et al.*, 2001). However, this was a comparison between young and older men and not including women. In this study, there was no significant correlation between rates of MPB and FSR in men or women. The acute 6-hour time frame of whole-body muscle protein breakdown, therefore, does not align to the averaged m. vastus lateralis FSR rates over 3-days in older men or women. This may be as a result of a potentially acute fed MPB rate and therefore, a matched FSR measure over the same period may have provided a better association than an integrated measure of MPS. As previously described, the individual values of FSR and MPB correspond with existing literature. However, with limited measures of MPB in current literature, further validation of the 3MH method would be required.

To observe relationships between these parameters, observing a change pre- and post- intervention where muscle mass is expected to change would be more beneficial. For example a positive correlation between myofibrillar MPS has been shown with muscle hypertrophy after resistance exercise (Damas *et al.*, 2015). This is because a high MPS rate does not necessarily result in a positive net protein turnover (i.e. where MPS>MPB) where an increase in muscle mass would be expected. As such, the measurement of net balance

is important. However, subtracting the MPB (%/h) from integrated MPS (%/h) in this study did not find a significant correlation with muscle mass. As mentioned previously, this may be due to the measure of cumulative FSR over a 3-day period of the m.vastus lateralis, not aligning with the measure of whole-body muscle protein breakdown over a 6-hour period.

The relationship between muscle size and strength has been well documented (Ikai and Fukunaga, 1970; Akagi *et al.*, 2009). Reed *et al.*, 1991 identified positive correlations between upper arm strength and midarm muscle area as well as leg strength and midthigh muscle area in the elderly (>65y, n=218). However, herein no significant correlations were found for men or women between whole-body muscle mass measured by DXA or D_3-Cr and 1-RM leg extension, although a slight trend was identified (males: p=0.067 and females: p=0.073). A significant correlation was found between whole-body muscle mass and knee extensor MVC for males, although it was not apparent in the females (p=0.075). This may be because of the lower n for females and therefore, underpowered. However, muscle strength does not solely depend on the size of the muscle. This is evident through studies showing improvements in muscle strength where muscle hypertrophy is no longer evident (M. S. Brook *et al.*, 2015) and additionally in longitudinal ageing studies where the loss of muscle strength occurs more rapidly than the loss of muscle mass (Delmonico *et al.*, 2009). This suggests that there are other

influencers e.g. neuromuscular or architectural (Atherton, Cegielski and Wilkinson, 2019).

Muscle architecture measures via ultrasound were also performed during this study. However, no significant correlations were observed between muscle architecture or VL thickness and muscle mass or leg muscle strength assessment via 1-RM or MVC. This may be as a result of only the VL thickness being measured and not be reflective of the whole quadriceps. Furthermore, the values presented are the average of both legs which may reduce the relationship with the single-leg strength measures.

Study limitations

As discussed, diet and physical activity was not monitored throughout the study. Although volunteers were asked to refrain from strenuous exercise, these factors were not monitored and can contribute to changes in MPS and MPB. For accurate assessment of D_3-Creatine spill over in the 24 h urine sample, this is reliant upon every pass of urine being collected. Adherence to this is difficult to monitor. Missed samples will reduce the total volume, thereby affecting total creatine concentration measures as well as missing the amount of unabsorbed D_3-Creatine that may be in that sample. The extent of the effect of a missed urine sample will differ between subjects based on their creatine excretions in 24h. For example, for a subject with a pooled 24h creatine concentration of 10 µg/ml vs a subject with 20 µg/ml, a 100 ml missed urine sample would have a greater effect on percentage dose for the subject with 20 µg/ml than 10 µg/ml. A 2%

dose spill over (i.e. 0.6mg of 30mg) can account for ~0.5kg increase in muscle mass if omitted. Therefore, additional measures such as 24h urinary creatinine excretion would have been beneficial to further investigate 24h urine completions.

Since it was the hypothesis of this study that the combined tracer methods may be employed as an approach to quantify muscle protein turnover and muscle mass, certain investigations in this chapter have employed multiple statistical comparisons testing to identify relationships between them. Multiple comparisons analysed independently of each other increase the probability of false positive discover (type I error), known as the familywise error rate (FWER). As a result, it is acknowledged that this study design may be susceptible to type I errors, having run multiple correlations independently. Future analysis may control the FWER by encompassing p-value adjustment methods. It should be noted however, that commonly employed approaches such as the Bonferroni and Benjamini-Hochberg methods assume tests are independent of each other. This is not the case in the comparison of different strength tests which can often be positively correlated, as observed here with muscle mass measures, and as such are unsuitable (Yoav and Hochberg, 1995; Miceli, 2012; Stevens, Masud and Suyundikov, 2017). Certain approaches such as the Holm and Benjamini & Yekutieli procedures which do not require independence of test statistics may prove useful (Holm, 1979; Benjamini and Yekutieli, 2001). Furthermore, in considering that any form of p-value

adjustment, intended at reducing type I errors, subsequently results in the inflation of type II errors (the probability of incorrectly accepting the null hypothesis)(Green and Britten, 1998). Since in this study the participant numbers were relatively low and the primary goal was to assess whether the combined tracer approach was a viable method to measure MPB, MPS and muscle mass concurrently; at the cost of a some false positives the potential benefits of this method suggest the potential to conduct future larger studies.

Future investigations

Further validation of the D_3-Creatine muscle method is required in a test-retest scenario with an intervention to identify if there are changes in the creatine pool size that could affect the method (e.g. exercise or feeding). However, at baseline the D_3-Creatine method is a more direct estimate of muscle mass and validations of this method in populations of low muscle mass (e.g. sarcopenic) should be made to identify if DXA underestimates the number of sarcopenic individuals. Further investigations into whether the D_3-creatine method would be able to detect differences in sarcopenic individuals based on current muscle mass and function criterion. Therefore, further testing would be required to identify the cut-off values for muscle mass when using the D_3-creatine method. The MPB results from this study identified scope in further investigating the sensitivity of the D_3-3MH method in detecting acute changes to muscle protein breakdown.

Conclusion

To conclude, this study has shown the feasibility of the triple isotopic tracer approach to estimate muscle mass, muscle protein synthesis and muscle protein breakdown simultaneously in humans, in a minimally invasive fashion. This could increase the possibility of MPS, MPB and muscle mass to be studied in more vulnerable populations (e.g. clinical or frail elderly). Although, no significant correlations were found between the muscle mass and muscle protein turnover measures for healthy elderly men and women, the individual measurements corresponded with previous literature. Therefore, this minimally invasive combined stable isotope approach can be applied for basal assessments.

6. A practical home-based intervention of a 4-week resistance exercise programme aimed at enhancing muscle mass and function in older adults: a pilot study

Cegielski Jessica[1], Brook Matthew S.[1], Quinlan Jonathan I.[1], Wilkinson Daniel J.[1], Smith Kenneth[1], Atherton Philip J.[1] and Phillips Bethan E[1].

This chapter is presented in manuscript format as published in F1000 Research. This manuscript is an open access article distributed under the terms of the Creative Commons Attribution Licence. This permits unrestricted use, distribution and reproduction in any medium, provided the original author and source are credited.

Figure 6.3.2.1 has been adapted for the purpose of this thesis.

Abstract:

Background: Developing alternative exercise programmes that can alleviate certain barriers to exercise; whether psychological, environmental or socio-economical, whilst providing similar physiological benefits e.g. increases in muscle mass and strength, is of grave importance. This pilot study aimed to assess the efficacy of an unsupervised, 4-week, whole-body home-based exercise training (HBET) programme, incorporated into daily living activities, on skeletal muscle mass, power and strength.

Methods: Twelve healthy older volunteers (63±3 years, 7 men: 5 women, BMI: 29±1 kg.m²) carried out the 4-week "lifestyle-integrated" HBET of 8 exercises, 3x 12 repetitions each, every day. Before and after HBET, a number of physical function tests were carried out: unilateral leg extension 1-RM (one- repetition maximum), MVC (maximal voluntary contraction) leg extension, lower leg muscle power (via Nottingham Power Rig), handgrip strength and SPPBT (short physical performance battery test). A D_3-Creatine method was used for assessment of whole-body skeletal muscle mass, and ultrasound was used to measure the quadriceps cross-sectional area (CSA) and *vastus lateralis* muscle thickness.

Results: Four weeks HBET elicited significant ($p<0.05$) improvements in leg muscle power (276.7±38.5 vs. 323.4±43.4 W), maximal voluntary contraction (60°: 154.2±18.4 vs. 168.8±15.2 Nm, 90°: 152.1±10.5 vs. 159.1±11.4 Nm) and quadriceps CSA (57.5±5.4 vs. 59.0 ± 5.3 mm), with a trend for an increase in leg strength (1RM: 45.7±5.9 vs. 49.6±6.0 Kg, P=0.08). This was despite

there being no significant differences in whole-body skeletal muscle mass, as assessed via D_3-Creatine.

Conclusions: This study demonstrates that increases in multiple aspects of muscle function can be achieved in older adults with just 4-weeks of "lifestyle-integrated" HBET, with a cost-effective means. This training mode may prove to be a beneficial alternative for maintaining and/or improving muscle mass and function in older adults.

6.1. Introduction

Older age is associated with the loss of skeletal muscle mass. Termed *sarcopenia*, age-related muscle wasting is associated with loss of muscle strength (*dynapenia*), increased morbidity, loss of independence and premature mortality (Rudrappa *et al.*, 2016). There are however proven means by which to offset these detrimental progressive declines. For example, structured and fully-supervised progressive resistance exercise training (RET) has been shown to improve both muscle mass (Phillips *et al.*, 2012) and function (Liu and Latham, 2009).

Despite the established effects of RET on muscle mass and function, compliance is difficult to achieve, due to perceived (and real) socio-economic, psychological and environmental factors (Zlot *et al.*, 2006). Almost all current evidence for the efficacy of RET is based on 2-3 sessions each week, for up to 12 weeks, at a gym or with specialist equipment, reflecting current physical activity guidelines for adults of 2-3 days per week RET for each of the major muscle groups (Bull *et*

al., 2010). However, compliance to physical activity recommendations is poor, with recent data suggesting that they are only achieved by 38% of UK adults (Department of Health, Physical Activity, 2011), a figure that is lesser still in older adults (Jefferis *et al.*, 2014). As a result, home-based exercise training (HBET) programmes have been suggested as an alternative to overcome some of the barriers associated with poor compliance to exercise (Silveira *et al.*, 2013; Hong *et al.*, 2016), whilst also providing a platform to develop the benefits of RET for (pre-) sarcopenic individuals (Maruya *et al.*, 2016).

The majority of HBET studies to date, have focussed on pre- or rehabilitation for specific clinical groups (Bassett and Prapavessis, 2007; Chang *et al.*, 2017). A small number of studies have specifically investigated the efficacy of HBET on skeletal muscle function in the elderly, although the focus of these studies has mainly been on lower limb exercises to improve walking and balance (Yamauchi, Nakayama and Ishii, 2009; Ito *et al.*, 2015) in relation to fall and fracture prevention. Other studies in older adults have incorporated the use of specialist aids e.g. dumbbells (Plotnikoff *et al.*, 2010), elastic bands (Jette *et al.*, 1999; Ribeiro *et al.*, 2009) or ankle weights (Gardner *et al.*, 2001) to provide resistance for HBET; rather than attempting to incorporate exercise into tasks of daily living, as is done herein. To date, no studies have attempted to investigate the efficacy of incorporating whole-body exercise into activities of daily living on muscle mass and function in older adults. Consequently, in this pilot

study we aimed to assess the effects of a 4-week, progressive, whole-body HBET, fully integrated into activities of daily living on muscle mass and function in healthy older individuals. Additionally, we aimed to utilise a recently re-introduced (Clark *et al*. 2014) and underexploited creatine method to estimate whole-body muscle mass in humans.

6.2. Materials and methods

6.2.1. Recruitment of study participants and ethical approval

Twelve healthy older male and female volunteers (63±3 y, 7 men: 5 women, BMI: 29±1 kg.m²) with no previous chronic disease history were recruited via an advertisement on a local radio station. Before enrolment into the study, all volunteers had an assessment of previous medical history and an ECG to check for any subclinical arrhythmias. The ECG's were approved by a clinically qualified physician. All volunteers provided written informed consent prior to the start of this study. This study was approved by The University of Nottingham Medical School Ethics Committee (B28102015 SoMS GEM BBC) and conformed to The Declaration of Helsinki.

6.2.2. Study procedures

The study consisted of two visits, before and after the 4-week HBET (Figure 1), both conducted at the University of Nottingham Medical School at the Royal Derby Hospital. All assessments at these visits

were conducted by researchers from the MRC-ARUK Centre for Musculoskeletal Ageing Research. At each visit, volunteers provided a baseline urine sample for the muscle mass assessment by D_3-Creatine before undergoing measures of height, weight (Marsden Weighing Group, UK), resting heart rate and blood pressure (Omron M5-1 digital BP monitor, Omron, UK). Each volunteer had the thickness, fascicle length (L_f) and pennation angle (θ_{pen}) of their *vastus lateralis* muscle*,* and the cross-sectional area (CSA) of their quadriceps measured by ultrasound (Mylab 70, Esaote Biomedica, Genova, Italy), using the protocol described by Franchi *et al*. (2014). Volunteers then completed a number of muscle function assessments:

- maximal voluntary contraction (MVC) for seated leg extension and flexion using an isokinetic dynamometer (Isocom; Isokinetic Technologies, Eurokinetics, UK),
- leg extension 1-repetition maximum (1-RM; ISO leg extension, Leisure Lines Ltd., GB),
- leg power (Nottingham Power Rig; Bassey & Short 1990),
- handgrip strength (Grip D 5401; Takei Scientific Instruments Co. Ltd., Japan); and
- a short physical performance battery test (SPPBT; Guralnik *et al*. 1994).

MVC was measured at two knee angles (60 and 90°), whilst isokinetic (eccentric/concentric) contractions were measured over a 70° range (20-90°) at three different speeds (60, 180 and 240°/sec). For both

measures, horizontal seated leg extension was set at 0°. Following the muscle function assessments, volunteers ingested 30mg of D_3-Creatine dissolved in 100ml H_2O for muscle mass assessment, before providing a pooled 24h urine collection and single urine samples at 48 and 72 h post consumption (Clark *et al.*, 2014).

6.2.3.Home-based exercise training programme (HBET)

Volunteers were required to complete 3 sets of 12 repetitions across 8 different exercises, every day, for 4 weeks. Each exercise was designed to be easily incorporated into activities of daily living to minimise the time-commitment required (e.g. bicep curls whilst cooking, see Figure 6.2.3.1). In total the exercise programme consisted of squats, lunges, calf raises, bicep curls, triceps extensions, semi-incline press-ups, oblique twists and deadlifts. Participants were instructed to take 2 seconds to perform each phase (eccentric and concentric) of each exercise and to hold for 2 seconds at the mid-point of each exercise. Progression via increased resistance or time-under-tension was encouraged once the full complement of repetitions could be completed. Compliance to exercise was measured by self-report (see self-report log in Figure 6.2.3.1). An exercise would only be marked as complete if 3 sets of 12 repetitions had been performed within a day.

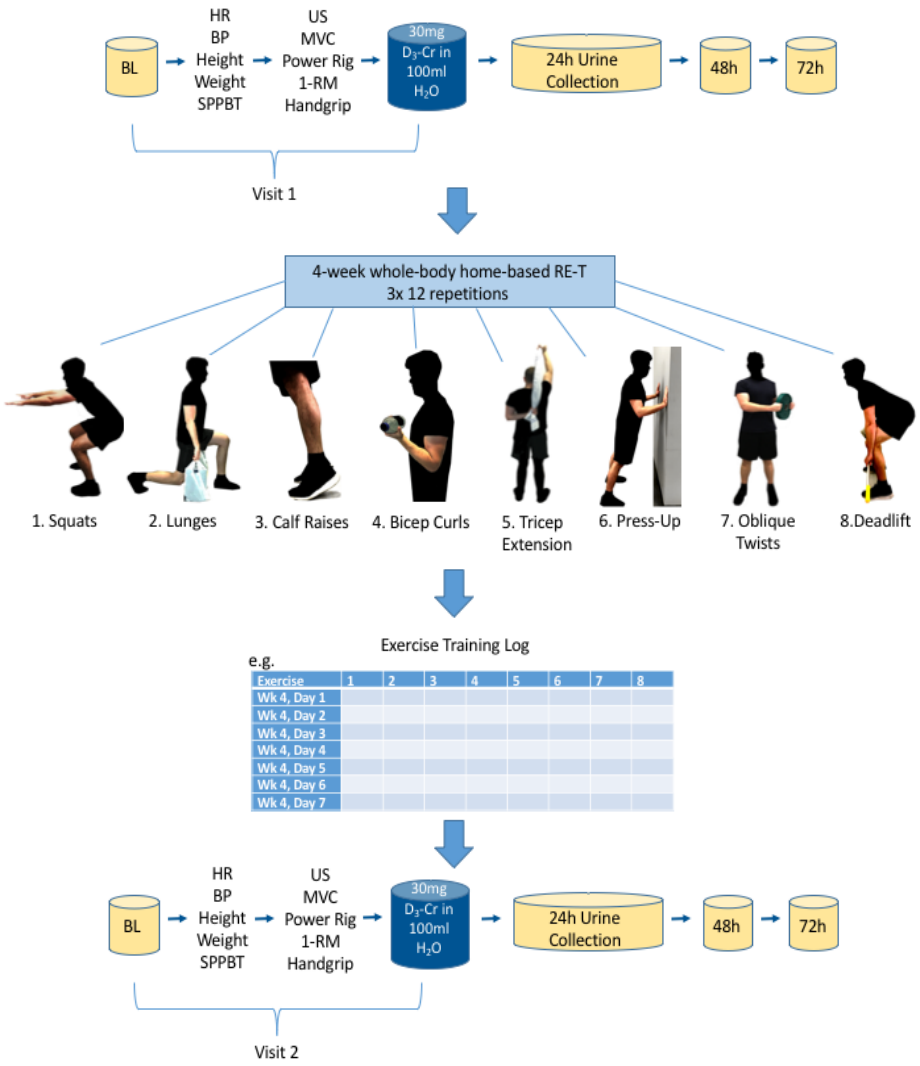


Figure 6.2.3.1 ***Study schematic displaying the two visits before and after home-based exercise training (HBET).*** *BL: baseline urine sample; HR: resting heart rate; BP: resting blood pressure; SPPBT: short physical performance battery test; US: leg ultrasound; MVC: maximum voluntary contraction; 1-RM: leg extension 1-repetition maximum; D_3-Cr: D_3-Creatine for whole-body muscle mass assessment; 48 and 72h represent the time of urine samples post-tracer consumption.*

6.2.4.Measurement of whole-body muscle mass using D_3-creatine

Using a creatine-tracer measure of muscle mass (Clark *et al.*, 2014), D_3-creatine and D_3-creatinine enrichment, plus unlabelled creatine and creatinine in the urine was measured using high-performance liquid chromatography/mass spectrometry (HPLC/MS), as detailed in

(Stimpson *et al*. 2012). Total muscle mass was calculated using the following equation:

$$\text{Total muscle mass} = \frac{\left(\frac{MW_{Unlabelled}}{MW_{labelled}}\right) \times (Amount\ of\ \ D_3{-}Cr\ dosed\ (g) - Amount\ of\ D_3{-}Cr\ excreted\ (g))}{(mean\ steady{-}state\ D_3{-}Creatinine\ enrichment\ ratio)} \div 4.3(g/kg)$$

(Clark *et al.*, 2014),

where $MW_{Unlabelled}$ and $MW_{labelled}$ represents the molecular weights of both unlabelled and labelled creatine, respectively. The estimated creatine pool size is then divided by 4.3 g/kg, which reflects the concentration of creatine in wet muscle.

6.2.5. Statistical analysis

Descriptive statistics were carried out on all datasets and checked for a normal distribution using the Shapiro-Wilk normality test. Datasets were analysed using paired Student's t test to assess the effect of HBET, with GraphPad Prism Software v5 (La Jolla, CA, USA). All data is presented as mean±SEM. Significance was set at $P<0.05$.

6.3. Results

6.3.1. Compliance, body weight and cardiovascular parameters

Using data from the self-report training logs, overall compliance to the HBET was 87.3±3.0% over the 4-week period of training. Adherence to each individual exercise was >86%, with the calf-raises being the most completed exercise (91%). No significant changes

were found following HBET for body weight (79.3±4.7 vs. 78.9±4.6 kg), resting heart rate (71.9±3.1 vs. 71.4±3.9 bpm) or blood pressure (systolic: 144.4±3.4 vs. 140.7±4.2, diastolic: 92.2±2.5 vs. 89.0±2.4; average of 3 measurements).

6.3.2.Muscle function

HBET did not elicit significant changes in leg extension 1-RM, handgrip strength or SPPBT; there was however, a trend for an increase in leg extension 1-RM (45.7±5.9 vs. 49.6±6.0 Kg, P=0.08; Figure 6.3.2.1A). HBET did elicit significant increases in leg power (276.7±38.5 vs. 323.4±43.4 W, P<0.05; Figure 6.3.2.1B) and MVC at 60° (154.2±18.4 vs. 168.8±15.2 Nm, P<0.05; Figure 6.3.2.1C) and 90° (152.1±10.5 vs. 159.1±11.4 Nm, P<0.05; Figure 6.3.2.1.D). There were also significant increases in peak torque recorded at 60°/sec during both seated flexion (74.7±8.3 vs. 80.8±8.6 Nm, P<0.05; Figure 5.3.2.1E) and extension (114.9±10.2 vs. 123.8±9.4 Nm, P<0.01; Figure 5.3.2.1F) after HBET.

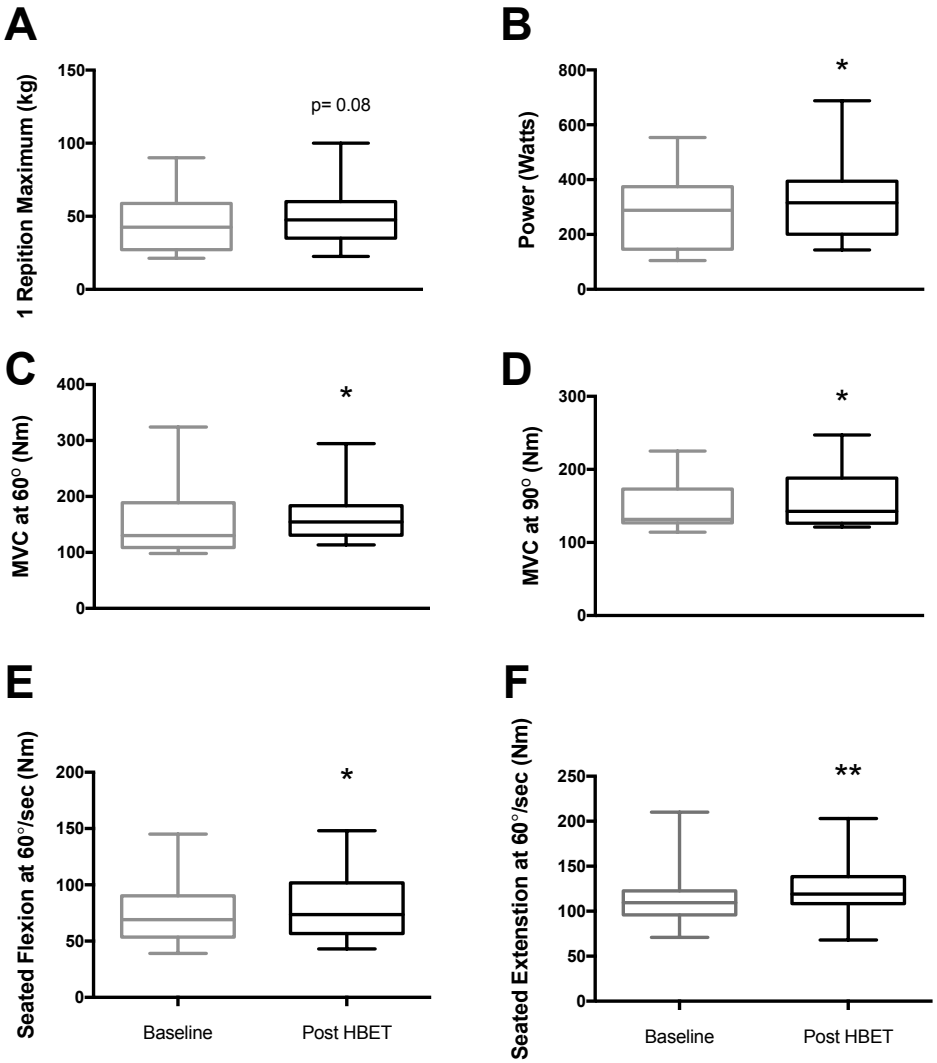


Figure 6.3.2.1 ***Muscle (leg) function assessments, before and after home-based exercise training (HBET).*** *A) 1-RM leg extension (45.7±5.9 vs. 49.6±6.0 Kg, P=0.08), B) Leg power via Nottingham Power Rig (276.7±38.5 vs. 323.4±43.4 W), C) MVC at 60° (154.2±18.4 vs. 168.8±15.2 Nm), D) MVC at 90° (152.1±10.5 vs. 159.1±11.4 Nm), E) Seated leg flexion at 60°/sec (74.7±8.3 vs. 80.8±8.6 Nm), F) Seated leg extension at 60°/sec (114.9±10.2 vs. 123.8±9.4 Nm). Baseline vs. post-HBET (mean ± SEM), n=12, *=p<0.05; **=p<0.01. MVC: Maximal voluntary contraction.*

6.3.3.Muscle architecture

CSA of the thigh at 50% of thigh length significantly increased (57.5±5.4 vs. 59.0 ± 5.3 mm, P<0.05; Figure 6.3.4.1.A) after HBET. However, there was no significant increase in muscle thickness, fascicle length (L_f) or pennation angle (θ_{pen}) of the *m. vastus lateralis*. There was a trend for an increase in muscle thickness (25.0±1.6 vs. 25.5±1.7 mm, P=0.08; Figure 6.3.4.1.B) after HBET.

6.3.4.Whole-body muscle mass measured by D_3-Creatine

Corresponding with no significant change in body weight, there was no significant difference in whole-body muscle mass as estimated by D_3-Creatine (Figure 6.3.4.1.C) after HBET (23.9±2.1 vs. 22.8±2.2 kg). There were no significant correlations between muscle mass measured by D_3-creatine and quadriceps CSA or VL thickness, pre or post HBET.

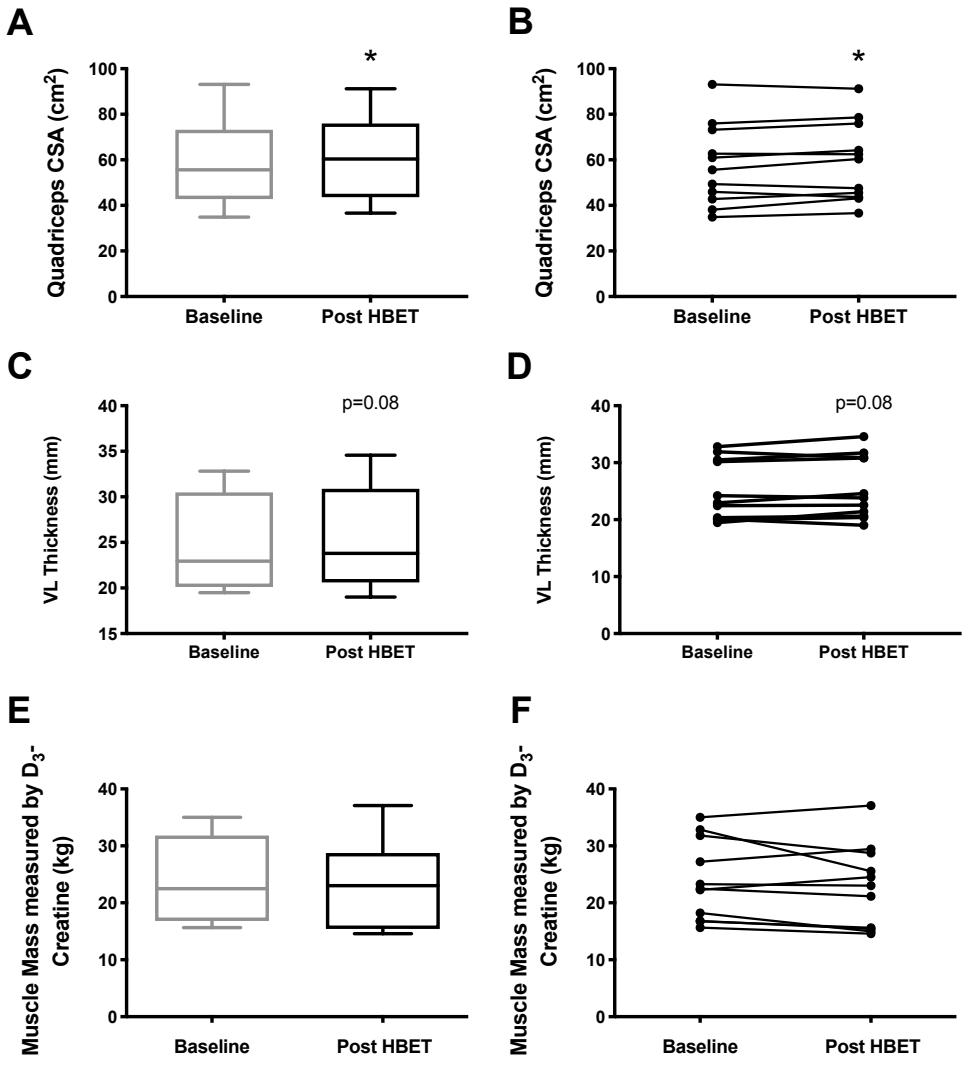


Figure 6.3.4.1 ***A) quadriceps cross-sectional area*** *(CSA; 57.5±5.4 vs. 59.0 ± 5.3 mm) with individual data points outlined in B);* ***C)*** **m. vastus lateralis** ***(VL) thickness*** *(25.0±1.6 vs. 25.5±1.7 mm, P=0.08 with individual data points outlined in D);* ***E) whole-body muscle mass, before and after home-based exercise training (HBET)*** *(23.9±2.1 vs. 22.8±2.2 kg) with individual data points outlined in F). (mean ± SEM), *=p<0.05, baseline vs. post-HBET.*

6.4. Discussion

In this pilot study, we show that lifestyle- integrated, whole-body HBET has the potential to meaningfully improve muscle function in older adults. We have shown that this training mode can increase leg power by 16.9% in just 4-weeks. Although lower than the 37% power increase demonstrated in a solely lower-extremity focused HBET programme, this study was conducted over an 8-week period (DeBolt

and McCubbin, 2004), suggesting that if the magnitude of increase was to continue at a constant rate, our training mode may elicit similar improvements. Additionally, we demonstrate increases in muscle strength (via MVC at 60°) similar to that observed in response to a 6-week, fully-supervised, gym-based training study (Brook *et al.*, 2016). Furthermore, despite no significant increase, there was a positive trend in the leg extension 1-RM, similar to that seen in an 8-week HBET study by Zion *et al*. (2003). Thus, while muscle function adaptations to HBET were numerically lesser compared to longer-term structured exercise regimens (some requiring specialist equipment), our data support the notion that just 4-weeks of a 'lifestyle-integrated' HBET meaningfully improves muscle function.

Despite these positive results, no changes in handgrip strength were found, a similar result to Nelson *et al*. (2004), who implemented a 6-month home-based whole-body exercise programme in 70 male and female elderly individuals. This may be due to a lack of exercises specifically targeting the forearm flexors. Indeed, although it is well-established that extant grip strength represents a biomarker of muscle function in older adults (Bassey and Harries, 1993), our data suggests that it is an insensitive biomarker for important functional and mass gains in other muscle groups (e.g. the legs) following certain exercise interventions.

Importantly, increases in thigh CSA (and a trend towards increased muscle thickness), which may partly explain the functional

improvements, were observed. Measures of thigh CSA by ultrasound have been positively correlated with those by MRI (Reeves, Maganaris and Narici, 2004), therefore suggesting (leg) muscle hypertrophy in this study. However, since this occurred in the absence of detectable changes in whole-body muscle mass as measured by D_3-Creatine, we speculate i) that preferential hypertrophy of leg muscles occurred, but remained undetectable on a whole-body level; or ii) that measurement of whole-body muscle mass was below the detection threshold for the D_3-creatine method.

We acknowledge limitations to our study design. The use of validated physical activity monitors (e.g. Actiheart, CamNtech, Cambridge, UK) to assess compliance to exercise would have provided greater information on the adoption of, and adherence to our exercise programme. Similarly, the use of interactive technologies (e.g. training applications for tablets/smartphones) for motivation and/or instruction may have improved adherence (Silveira *et al.*, 2013). The lack of improvements in SPPBT were likely a result of our volunteers achieving optimal scores before the intervention.

To conclude, our findings demonstrate that significant increases in muscle mass, maximal voluntary contraction, maximal power and isokinetic strength can be achieved with just 4-weeks of unsupervised, lifestyle-integrated HBET in older adults. These improvements were achieved via cost-effective means, without the requirement for specialist equipment, facilities or supervision (Jette

et al., 1996; Zion *et al.*, 2003; DeBolt and McCubbin, 2004). With high compliance and beneficial physiological effects in only a 4-week period, this study highlights the potential for lifestyle-integrated HBET to improve skeletal muscle 'health' in older adults. As increases in muscle mass and strength are positively correlated with overall physical function (Liu and Latham, 2009), this novel training mode may be of particular benefit to older frail/ (pre-) sarcopenic adults.

7. Overall Discussion

It is expected that 1 in 4 people in the UK will be aged over 65 years by 2050 (Office for National Statistics, 2018). With ageing there is loss of muscle mass, strength and function, which collectively has been termed as sarcopenia (Cruz-Jentoft *et al.*, 2010). Sarcopenia is associated with an increased risk of falls (Gschwind *et al.*, 2013), bone fractures (Tarantino *et al.*, 2015) and co-morbidities (Tieland, Trouwborst and Clark, 2017), which ultimately leads to a reduced quality of life. Muscle mass is determined via a dynamic equilibrium between MPS and MPB (S M Phillips, Glover and Rennie, 2009). Thus, when screening for the efficacy of any form of intervention (e.g. nutritional, pharmacological and/or exercise) to mitigate muscle loss in old age it is crucial to consider both MPS and MPB.

Traditional techniques used to measure MPS or MPB are relatively invasive; requiring the use of intravenous infusions, cannulation and multiple biopsies (Wilkinson *et al.*, 2015). Furthermore, imaging techniques such as MRI/CT or DXA to assess muscle mass typically may have limited accessibility. The above techniques pose additional logistical challenges when applied to more vulnerable populations such as, care homes or frail elderly where sarcopenia is more likely to be evident. Therefore, the development of more minimally invasive techniques that have the ability to be reproduced in such populations is essential to further our understanding of sarcopenia. Nonetheless,

the recent re-introduction of the labelled creatine method for assessment of muscle mass by Clark et al. (2014) and the use of D_3-3-methylhistidine for the assessment of whole-body breakdown by Sheffield-Moore et. al (2014) may provide more accessible alternatives. Significantly, these considerably less invasive techniques may be used to investigate muscle mass and MPB in broader populations.

Therefore, the aims of this PhD thesis were threefold. Firstly, to investigate the research population of older individuals to determine how effective current methods are in the identification of sarcopenia. Secondly, the development and feasibility of the D_3-creatine and D_3-3-methylhistidine methods and to identify a protocol in which these tracer methods could be used alongside D_2O; allowing for MPS, MPB and muscle mass simultaneously. Thirdly, to use this approach in older individuals to identify any relationships between the three measures within the older demographic.

Herein, we have developed a novel approach combining three minimally invasive stable isotope tracer techniques to measure MPS, MPB and muscle mass concurrently. The results of the combined use of deuterium oxide, D_3-3-methylhistidine, and D_3-creatine for the assessment of MPS, MPB and muscle mass respectively, align with previous individual validation studies using these techniques (Clark *et al.*, 2014; Sheffield-Moore *et al.*, 2014; Wilkinson *et al.*, 2014). In addition, we also assessed the use of the D_3-3MH tracer and D_3-

creatine tracer alongside interventions where an expected change in muscle mass. Although in the 4-week HBET this would not have been sufficient, but the main aim in that study was to assess the feasibility of the exercise programme. Currently, due to the previous difficulty of measuring MPB, the effects of disuse atrophy on muscle proteolysis remains unclear (Rudrappa *et al.*, 2016). Therefore, the D_3-3MH tracer was applied pre- and post- 7-day bed rest in young males. In contrast to a study by Symons et al. (2009) who found no significant difference in the rate of MPB after 21 days of simulated microgravity (Symons *et al.*, 2009), herein we identified a suppression in whole-body MPB. Our findings are in agreement with the suggestion that a suppression in muscle protein synthesis (i.e. anabolic blunting) along with a lowered MPB exists after immobilization (S. M. Phillips, Glover and Rennie, 2009). However, more research is required to further understand the role of MPB on disuse atrophy (Crossland *et al.*, 2019).

From these promising results we applied the combined oral stable isotope assessment of muscle (COSIAM) approach to a healthy elderly population to identify if there were any gender differences or if the use of these techniques could uncover any novel relationships between MPS, MPB and muscle mass.

In this second study, we found that the D_3-creatine dose spill over was higher in the females than in the males. This is in agreement with the findings from the study by Clark et al. (2018), where the median excretion was 3.46% vs 25.63% for men vs women, respectively.

This highlighted that the use of the 24-hour pooled sample would still be vital in females, limiting the ability of this method to being reduced to a single urine sample post-tracer ingestion. Despite this difference, there was still a strong correlation between the assessment of muscle mass by DXA and D_3-creatine for males and females, consistent with current literature (Clark *et al.*, 2014, 2018; Shankaran *et al.*, 2018). With the combination of these approaches, no significant correlations were identified between MPS or MPB with muscle mass or function. However, this approach was applied only as a single measure, further investigations using COSIAM in an approach where a change in muscle mass is likely (i.e. via an exercise intervention or immobilisation) may identify relationships such as percentage change in muscle vs. percentage change in net balance.

This approach did however, identify a significant correlation between muscle leg power and muscle mass measured by D_3-Creatine in the older males ($p=0.049$) and some close to significant correlations between muscle mass and muscle strength (handgrip strength: $p=0.078$, MVC: $p=0.075$) in the older females and in the older males (single-leg 1RM leg extension: $p=0.067$). When both male and female data was combined, significant positive correlations ($p<0.0001$) exist between muscle mass measured by D_3-creatine and handgrip strength ($r=0.71$), single leg 1RM leg extension ($r=0.72$), single leg MVC ($r=0.70$) and leg power ($r=0.64$). These results support the recent study in 1,382 older men whereby muscle mass determined by the D_3-creatine method significantly correlated with measures of physical function and mobility (Cawthon *et al.*, 2019).

The COSIAM approach developed in this study has the potential to be further adapted to become even less invasive, such as through the substitution of urine sampling for baseline enrichment in place of a blood sample. Thereby, providing an additional alternative ability to investigate muscle protein turnover and muscle mass in less accessible populations e.g. care homes/ frail elderly or clinical.

However, we do acknowledge that this study has limitations. Whilst participant's dietary habits and physical activity was discussed during the screening visit, neither aspect was monitored during the COSIAM approach. Although our meat vs meatless diets found no significant rates in the measure of MPB using D_3-3MH (Sheffield-Moore et al. 2014), the use of creatine supplementation or vegetarian/ vegan based diets may increase or decrease the creatine pool size respectively. Consequently, if the pool is influenced by external factors, the measure of muscle mass may in fact be an over or underestimation as the calculation is based on an averaged 4.3 g of creatine / kg of wet weight muscle mass. However, the creatine pool size of the muscle was not measured in this study to assess any variability between men and women or to assess its stability. Therefore, future work is required to investigate the creatine pool size to identify if 4.3 g/kg is a representative coefficient across multiple populations. Significantly, a recent study by Morris-Paterson et al. (2019) identified that an assumed value of 5.1g/kg aligned the muscle mass measures by D_3-Creatine to the values of MRI in highly trained kayaking athletes, despite not measuring the creatine pool

size. Changes in creatine pool size may also be affected by changes in fibre-type composition (Brault, Abraham and Terjung, 2003). As muscle fibre composition changes with age (Lexell, Taylor and Sj, 1988), investigations into whether the change significantly alters the 4.3 g/kg constant are required. Furthermore, from results herein, future investigations into the sensitivity and reproducibility of D_3-3MH method to assess changes in whole-body MPB over an acute time frame should be made as there may be potential for the use of this tracer in a broader set of study designs.

We further investigated the use of D_3-creatine in the assessment of muscle mass pre- and post- intervention. Resistance exercise is recommended as a treatment for sarcopenia (Peterson and Gordon, 2011). However, adherence to regular resistance exercise can be difficult with a number of socioeconomic, psychological and environmental factors (e.g. access to specialised training equipment) or "lack of time"; one of the most commonly cited barriers (Trost *et al.*, 2002). Therefore, we aimed to develop a home-based exercise training (HBET) programme that was integrated into activities of daily living as a potential solution to mitigating some of the barriers surrounding resistance exercise, whilst aiming to observe the effect of HBET on muscle mass, muscle strength and function. The D_3-creatine tracer did not identify any changes in muscle mass, which may be due to an insufficient intensity of the exercise. The main aim of the study was the development of a feasible home-based exercise program which was the main focus, therefore, it may not have been the most ideal situation in which to test D_3-creatine as it was low

intensity and in a short-time frame. A limitation to this study is that no DXA or MRI was performed to assess any whole-body muscle mass changes that could support the findings of the D_3-creatine tracer. Further testing into the applicability of the D_3-creatine tracer in a test-retest situation is required.

The "gold standard" for measuring muscle mass is MRI (Maden-Wilkinson *et al.*, 2013), which is expensive and accessibility can be limited. An alternative imaging technique that is commonly used for the estimation of muscle mass, including for the identification of sarcopenic individuals, is DXA. However, DXA does not measure muscle mass directly as it provides a calculation of lean body mass. This calculation is a combination of connective tissue, muscle mass, organ tissue and water weight (Evans *et al.*, 2019) and thus, not a direct measure of muscle mass. A number of definitions for sarcopenia utilise DXA as a technique to quantify low muscle mass. As DXA does not measure muscle mass directly, this may result in an underestimation of the number of sarcopenic individuals in the population. The updated definition by the European Working Group on Sarcopenia in Older People (EWGSOP2) has acknowledged the recent developments and potential of the D_3-creatine method for the assessment of muscle mass, however, further validation of the method in greater populations is required (Cruz-Jentoft *et al.*, 2018). Despite the potential of the D_3-creatine method to assess low muscle mass and identify sarcopenic individuals, we have highlighted a vast number of discrepancies in the current definitions used for sarcopenia identification. These exist not only for the quantification of low muscle

mass but also for the determination of sarcopenia based on muscle strength or functional assessments. We identify that the four main definitions of sarcopenia (by the EWGSOP, EWGSOP2, IWGS and FNIH) identify different prevalence of sarcopenia in our cohort. This is critical as depending on the definition of sarcopenia used, the prevalence may be either under or over reported. For future identification and adequate treatment provision an agreement on a coherent single definition of sarcopenia should be made. A collective database of numerous studies of muscle mass across age should be formulated with the aim of developing T-scores as similarly used in the identification of BMD or as used to determine the handgrip strength cutoffs (Dodds *et al.*, 2014). A consistent definition will aid clinicians in the correct identification of sarcopenia and subsequent treatment plans.

To conclude, we have developed and demonstrated the use of a combined stable isotope approach to measure MPS, MPB and muscle mass in a minimally invasive fashion. We further applied this approach to a cohort of older adults and identified relationships between muscle mass measured using the D_3-creatine method and muscle strength. Additionally, we have identified the potential of the D_3-creatine method and D_3-3MH method to be used to assess changes in muscle mass and MPB, respectively in intervention studies. Although, the efficacy of the tracers needs to be investigated further. However, this combined minimally invasive stable isotope approach has the potential to be used in wider populations such as frail elderly/

care homes or clinical and aid research studies investigating sarcopenia. Thus, we investigated the current consensus on sarcopenia and identified a large disparity between the different definitions used and how sarcopenia is identified. Thus, a single consensus and definition of sarcopenia to provide coherent identification amongst clinicians and researchers.

References

Abdulla, H. *et al.* (2016) 'Role of insulin in the regulation of human skeletal muscle protein synthesis and breakdown: a systematic review and meta-analysis', *Diabetologia*, 59(1), pp. 44–55. doi: 10.1007/s00125-015-3751-0.

Abu-Zidan, F. M., Hefny, A. F. and Corr, P. (2011) 'Clinical ultrasound physics', *Journal of emergencies, trauma, and shock*. Medknow Publications, 4(4), pp. 501–503. doi: 10.4103/0974-2700.86646.

Akagi, R. *et al.* (2009) 'Muscle volume compared to cross-sectional area is more appropriate for evaluating muscle strength in young and elderly individuals', *Age and Ageing*, 38(5), pp. 564–569. doi: 10.1093/ageing/afp122.

Anker, S. D., Morley, J. E. and von Haehling, S. (2016) 'Welcome to the ICD-10 code for sarcopenia', *Journal of Cachexia, Sarcopenia and Muscle*, 7(5), pp. 512–514. doi: 10.1002/jcsm.12147.

Anker, S. D. and Sharma, R. (2002) 'The syndrome of cardiac cachexia', *International Journal of Cardiology*, 85(1), pp. 51–66. doi: 10.1016/S0167-5273(02)00233-4.

Asatoor, A. M. and Armstrong, M. D. (1967) '3-Methylhistidine, a component of actin', *Biochemical and Biophysical Research Communications*, 26(2), pp. 168–174. doi: 10.1016/0006-291X(67)90229-X.

Atherton, Philip J, Smith, K., *et al.* (2010) 'Distinct anabolic signalling responses to amino acids in C2C12 skeletal muscle cells.', *Amino acids*, 38(5), pp. 1533–9. doi: 10.1007/s00726-009-0377-x.

Atherton, Philip J, Etheridge, T., *et al.* (2010) 'Muscle full effect after oral protein : time-dependent concordance and discordance between human muscle protein synthesis and mTORC1', *The American journal of clinical nutrition*, (2), pp. 1080–1088. doi: 10.3945/ajcn.2010.29819.1.

Atherton, Philip J. *et al.* (2010) 'Muscle full effect after oral protein: Time-dependent concordance and discordance between human muscle protein synthesis and mTORC1 signaling', *American Journal of Clinical Nutrition*, 92(5), pp. 1080–1088. doi: 10.3945/ajcn.2010.29819.

Atherton, P. J. *et al.* (2016) 'Control of Skeletal Muscle Atrophy in Response to Disuse: Clinical/Preclinical Contentions and Fallacies of Evidence', *American Journal of Physiology - Endocrinology And Metabolism*, p. ajpendo.00257.2016. doi: 10.1152/ajpendo.00257.2016.

Atherton, P. J., Cegielski, J. and Wilkinson, D. J. (2019) 'Genetic contributions to skeletal muscle size', *Routledge Handbook of Sport and Exercise Systems Genetics*, pp. 277–289. doi: 10.4324/9781315146287-20.

Atherton, P. J. and Smith, K. (2012) 'Muscle protein synthesis in response to nutrition and exercise.', *The Journal of physiology*, 590(Pt 5), pp. 1049–57. doi: 10.1113/jphysiol.2011.225003.

Baldwin, B. D. *et al.* (1952) 'INTERRELATIONS OF MAGNESIUM , POTASSIUM , PHOSPHORUS , AND CREATINE IN SKELETAL MUSCLE

OF MAN ’ In latter years the growth of methods has led to a detailed understanding of the concentration and distribution patterns of electrolytes and water in the extra’, pp. 850–858.
Balsom, P. D., Söderlund, K. and Ekblom, B. (1994) ‘Creatine in Humans with Special Reference to Creatine Supplementation’, *Sports Medicine*, 18(4), pp. 268–280. doi: 10.2165/00007256-199418040-00005.
De Bandt, J.-P. and Cynober, L. (2006) ‘Therapeutic use of branched-chain amino acids in burn, trauma, and sepsis.’, *The Journal of nutrition*, 136, pp. 308S–13S. doi: 136/1/308S [pii].
Bárány, M. (1967) ‘ATPase activity of myosin correlated with speed of muscle shortening.’, *The Journal of general physiology*, 50(6). doi: 10.1085/jgp.50.6.197.
Barnes, B. B. A., Gordon, E. B. and Cope, O. (1957) ‘SKELETAL MUSCLE ANALYSES IN HEALTH AND IN CERTAIN METABOLIC DISORDERS . I . THE METHOD OF ANALYSIS AND THE VALUES IN NORMAL MUSCLE ’ Hospital , Boston , Mass .) Tissue analyses are of special importance in re- gard to the meager information available toda’, pp. 1239–1248.
Bassett, S. F. and Prapavessis, H. (2007) ‘Home-based physical therapy intervention with adherence-enhancing strategies versus clinic-based management for patients with ankle sprains.’, *Physical therapy*, 87(9), pp. 1132–43. doi: 10.2522/ptj.20060260.
Bassey, E. J. and Short, A. H. (1990) ‘A new method for measuring power output in a single leg extension: feasibility, reliability and validity’, *European journal of applied physiology*, 60, pp. 385–390.
Bassey, U. and Harries (1993) ‘Normal values for handgrip strength in 920 men and women aged over 65 years, and longitudinal changes over 4 years in 620 survivors’, *Clinical Science*, 84, pp. 331–337. doi: 10.1042/cs0840331.
Baumgartner, R. N. *et al.* (1998) ‘Epidemiology of sarcopenia among the elderly in New Mexico.[Erratum appears in Am J Epidemiol 1999 Jun 15;149(12):1161]’, *American Journal of Epidemiology*, 147(8), pp. 755–763.
Bederman, I. R. *et al.* (2006) ‘Novel application of the "doubly labeled" water method: measuring CO2 production and the tissue-specific dynamics of lipid and protein in vivo.’, *American journal of physiology. Endocrinology and metabolism*, 290(5), pp. E1048–E1056. doi: 10.1152/ajpendo.00340.2005.
Benjamini, Y. and Yekutieli, D. (2001) ‘The control of the false discovery rate in multiple testing under dependency’, *Annals of Statistics*, 29(4), pp. 1165–1188. doi: 10.1214/aos/1013699998.
Bernabeu-Wittel, M. *et al.* (2019) ‘Impact of Sarcopenia and Frailty in a Multicenter Cohort of Polypathological Patients’, *Journal of Clinical Medicine*, 8(4), p. 535. doi: 10.3390/jcm8040535.
Bevier, W. C. *et al.* (1989) ‘Relationship of body composition, muscle strength, and aerobic capacity to bone mineral density in older men and women’, *Journal of Bone and Mineral Research*, 4(3), pp. 421–432. doi: 10.1002/jbmr.5650040318.
Beynon, R. J. and Pratt, J. M. (2005) ‘Metabolic labeling of proteins for proteomics.’, *Molecular & cellular proteomics : MCP*, 4(7), pp. 857–72. doi: 10.1074/mcp.R400010-MCP200.

Bhasin, S. *et al.* (1996) 'The effects of supraphysiologic doses of testosterone on muscle size and strength in normal men', *New England Journal of Medicine*, 335(1), pp. 1–7. doi: 10.1056/NEJM199607043350101.
Blancquaert, L. *et al.* (2018) 'Changing to a vegetarian diet reduces the body creatine pool in omnivorous women, but appears not to affect carnitine and carnosine homeostasis: A randomised trial', *British Journal of Nutrition*, 119(7), pp. 759–770. doi: 10.1017/S000711451800017X.
Bodine, S. C. *et al.* (2001) 'Identification of ubiquitin ligases required for skeletal Muscle Atrophy', *Science*, 294(5547), pp. 1704–1708. doi: 10.1126/science.1065874.
Bohannon, R. W. (2008) 'Hand-grip dynamometry predicts future outcomes in aging adults', *Journal of Geriatric Physical Therapy*, 31(1), pp. 3–10. doi: 10.1519/00139143-200831010-00002.
Bohé, J. *et al.* (2001) 'Latency and duration of stimulation of human muscle protein synthesis during continuous infusion of amino acids.', *The Journal of physiology*, 532(Pt 2), pp. 575–9.
Borkan, G. A. *et al.* (1983) 'Age changes in body composition revealed by computed tomography', *Journals of Gerontology*, 38(6), pp. 673–677. doi: 10.1093/geronj/38.6.673.
Brault, J. J., Abraham, K. A. and Terjung, R. L. (2003) 'Muscle creatine uptake and creatine transporter expression in response to creatine supplementation and depletion', *Journal of Applied Physiology*, 94(6), pp. 2173–2180. doi: 10.1152/japplphysiol.01171.2002.
Broek, R. W. TEN, Grefte, S. and Von Den Hoff, J. W. (2010) 'Regulatory Factors and Cell Populations Involved in Skeletal Muscle Regeneration', *Journal of Cellular Physiology*, 224, pp. 7–16. doi: 10.1002/jcp.22127.
Brook, Matthew S. *et al.* (2015) 'Skeletal muscle hypertrophy adaptations predominate in the early stages of resistance exercise training, matching deuterium oxide-derived measures of muscle protein synthesis and mechanistic target of rapamycin complex 1 signaling', *FASEB Journal*, 29(11), pp. 4485–4496. doi: 10.1096/fj.15-273755.
Brook, M. S. *et al.* (2015) 'Skeletal muscle hypertrophy adaptations predominate in the early stages of resistance exercise training, matching deuterium oxide-derived measures of muscle protein synthesis and mechanistic target of rapamycin complex 1 signaling', *The FASEB Journal*, pp. 1–12. doi: 10.1096/fj.15-273755.
Brook, M. S. *et al.* (2016) 'Synchronous deficits in cumulative muscle protein synthesis and ribosomal biogenesis underlie age-related anabolic resistance to exercise in humans', *The Journal of Physiology*, 00, pp. 1–19. doi: 10.1113/JP272857.
Bull, F. C. *et al.* (2010) 'Physical activity guidelines in the U.K.: Review and Recommendations', *School of Sport, Exercise and Health Sciences, Loughborough University*, May, pp. 1–72.
Burd, N. A. *et al.* (2010) 'Low-load high volume resistance exercise stimulates muscle protein synthesis more than high-load low volume resistance exercise in young men.', *PloS one*, 5(8), p. e12033. doi: 10.1371/journal.pone.0012033.

Burd, N. a *et al.* (2011) 'Validation of a single biopsy approach and bolus protein feeding to determine myofibrillar protein synthesis in stable isotope tracer studies in humans.', *Nutrition & metabolism*. BioMed Central Ltd, 8(1), p. 15. doi: 10.1186/1743-7075-8-15.
Burke, R. and Edgerton, R. V (1975) 'Motor Unit Properties and Selective Involvement in Movement', *Exercise and sport sciences reviews*, 3(1), pp. 31–82.
Busch, R. *et al.* (2006) 'Measurement of protein turnover rates by heavy water labeling of nonessential amino acids.', *Biochimica et biophysica acta*, 1760(5), pp. 730–44. doi: 10.1016/j.bbagen.2005.12.023.
Busch, R. *et al.* (2008) 'Heavy water labeling of DNA for measurement of cell proliferation and recruitment during primary murine lymph node responses against model antigens.', *Journal of immunological methods*, 337(1), pp. 24–34. doi: 10.1016/j.jim.2008.05.014.
Buse, M. G. and Reid, S. S. (1975) 'Leucine. A possible regulator of protein turnover in muscle', *Journal of Clinical Investigation*, 56(5), pp. 1250–1261. doi: 10.1172/JCI108201.
Candow, D. G. and Chilibeck, P. D. (2005) 'Differences in size, strength, and power of upper and lower body muscle groups in young and older men', *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*, 60(2), pp. 148–156. doi: 10.1093/gerona/60.2.148.
Cao, L. and Morley, J. E. (2016) 'Sarcopenia Is Recognized as an Independent Condition by an International Classification of Disease, Tenth Revision, Clinical Modification (ICD-10-CM) Code', *Journal of the American Medical Directors Association*. AMDA – The Society for Post-Acute and Long-Term Care Medicine, 17(8), pp. 675–677. doi: 10.1016/j.jamda.2016.06.001.
Carter, J. and Barwick, V. (2011) *Good Practice Guide for Isotope Ratio Mass Spectrometry*. 1st edn. Edited by J. Carter and V. Barwick. FIRMS.
Cawthon, P. M. *et al.* (2019) 'Strong relation between muscle mass determined by d3-creatine dilution, physical performance, and incidence of falls and mobility limitations in a prospective cohort of older men', *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*, 74(6), pp. 844–852. doi: 10.1093/gerona/gly129.
Chang, C.-F. *et al.* (2017) 'Effects of a Home-Based Resistance Training Program on Recovery From Total Hip Replacement Surgery: Feasibility and Pilot Testing.', *Journal of Nursing Research*, 25(1), pp. 21–30.
Chargé, S. B. P. and Rudnicki, M. a (2004) 'Cellular and molecular regulation of muscle regeneration.', *Physiological reviews*, 84(1), pp. 209–38. doi: 10.1152/physrev.00019.2003.
Chen, L. K. *et al.* (2014) 'Sarcopenia in Asia: Consensus report of the Asian working group for sarcopenia', *Journal of the American Medical Directors Association*. Elsevier Ltd, 15(2), pp. 95–101. doi: 10.1016/j.jamda.2013.11.025.
Chen, Z. *et al.* (2007) 'Dual-energy X-ray absorptiometry is a valid tool for assessing skeletal muscle mass in older women', *J Nutr*,

137(1541-6100 (Electronic)), pp. 2775–2780. doi: 137/12/2775 [pii].
Chumlea, W. C. *et al.* (1999) 'Total body water data for white adults 18 to 64 years of age: The Fels Longitudinal Study', *Kidney International*, 56(1), pp. 244–252. doi: 10.1046/j.1523-1755.1999.00532.x.
Churchward-Venne, T. A. *et al.* (2015) 'There are no nonresponders to resistance-type exercise training inolder men and women', *Journal of the American Medical Directors Association*. Elsevier Inc., 16(5), pp. 400–411. doi: 10.1016/j.jamda.2015.01.071.
Clark, R. V. *et al.* (2018) 'Creatine (methyl-d 3) dilution in urine for estimation of total body skeletal muscle mass-accuracy and variability vs. MRI and DXA', *Journal of Applied Physiology*, 124(1–9), p. jap.00455.2016. doi: 10.1152/japplphysiol.00455.2016.
Clark, R. V *et al.* (2014) 'Total body skeletal muscle mass: estimation by creatine (methyl-d3) dilution in humans.', *Journal of Applied Physiology*, 116(12), pp. 1605–13. doi: 10.1152/japplphysiol.00045.2014.
Collins, M. L. *et al.* (2003) 'Measurement of mitochondrial DNA synthesis in vivo using a stable isotope-mass spectrometric technique.', *Journal of applied physiology (Bethesda, Md. : 1985)*, 94(6), pp. 2203–11. doi: 10.1152/japplphysiol.00691.2002.
Committee on Medical Aspects of Food Policy (1991) 'Dietary reference values for food energy and nutrients for the United Kingdom. Report of the Panel on Dietary Reference Values of the Committee on Medical Aspects of Food Policy.', *Reports on health and social subjects*. England, 41, pp. 1–210.
Crossland, H. *et al.* (2019) 'The impact of immobilisation and inflammation on the regulation of muscle mass and insulin resistance: different routes to similar end-points', *Journal of Physiology*, 597(5), pp. 1259–1270. doi: 10.1113/JP275444.
Cruz-Jentoft, A. J. *et al.* (2010) 'Sarcopenia: European consensus on definition and diagnosis: Report of the European Working Group on Sarcopenia in Older People.', *Age and ageing*, 39(4), pp. 412–23. doi: 10.1093/ageing/afq034.
Cruz-Jentoft, A. J. *et al.* (2018) 'Sarcopenia: European consensus on definition and diagnosis', *Age and Ageing*, 0, pp. 1–16. doi: 10.1093/ageing/afq169.
Cruz-Jentoft, A. J. and Sayer, A. A. (2019) 'Sarcopenia', *The Lancet*, 393(10191), pp. 2636–2646. doi: 10.1016/S0140-6736(19)31138-9.
Cuthbertson, D. *et al.* (2005) 'Anabolic signaling deficits underlie amino acid resistance of wasting, aging muscle.', *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 19(3), pp. 422–4. doi: 10.1096/fj.04-2640fje.
Damas, F. *et al.* (2015) 'A review of resistance training-induced changes in skeletal muscle protein synthesis and their contribution to hypertrophy.', *Sports medicine (Auckland, N.Z.)*, 45(6), pp. 801–7. doi: 10.1007/s40279-015-0320-0.
Davies, D. S. C. *et al.* (2019) 'UK Chief Medical Officers' Physical Activity Guidelines', *Department of Health and Social Care*, (September), pp. 1–65.

DeBolt, L. S. and McCubbin, J. A. (2004) 'The Effects of Home-Based Resistance Exercise on Balance, Power, and Mobility in Adults with Multiple Sclerosis', *Archives of Physical Medicine and Rehabilitation*, 85(2), pp. 290–297. doi: 10.1016/j.apmr.2003.06.003.
Defoiche, J. *et al.* (2009) 'Measurement of ribosomal RNA turnover in vivo by use of deuterium-labeled glucose.', *Clinical chemistry*, 55(10), pp. 1824–33. doi: 10.1373/clinchem.2008.119446.
Delmonico, M. J. *et al.* (2009) 'Longitudinal study of muscle strength, quality, and adipose tissue infiltration', *American Journal of Clinical Nutrition*, 90(6), pp. 1579–1585. doi: 10.3945/ajcn.2009.28047.
Deng, H. W. *et al.* (2002) 'Differences in bone mineral density, bone mineral content, and bone areal size in fracturing and non-fracturing women, and their interrelationships at the spine and hip', *Journal of Bone and Mineral Metabolism*, 20(6), pp. 358–366. doi: 10.1007/s007740200052.
Department of Health, Physical Activity, H. I. and P. (2011) *Start Active, Stay Active - A report on physical activity for health from the four home countries' Chief Medical Officers*, *Report*. doi: https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/216370/dh_128210.pdf.
Diamond, T. and Sheu, A. (2016) 'Bone mineral density: testing for osteoporosis', *Australian Prescriber*, 39(2), pp. 35–39.
Dideriksen, K. *et al.* (2020) 'Muscle protein breakdown is impaired during immobilization compared to during a subsequent retraining period in older men: no effect of anti-inflammatory medication', *Pflugers Archiv European Journal of Physiology*. Pflügers Archiv - European Journal of Physiology, 472(2), pp. 281–292. doi: 10.1007/s00424-020-02353-w.
Dodds, R. M. *et al.* (2014) 'Grip strength across the life course: Normative data from twelve British studies', *PLoS ONE*, 9(12), pp. 1–15. doi: 10.1371/journal.pone.0113637.
Drummond, M. J. *et al.* (2009) 'Regulation of Protein Metabolism in Exercise and Recovery Expression of growth-related genes in young and older human skeletal muscle following an acute stimulation of protein synthesis', 1144, pp. 1403–1411. doi: 10.1152/japplphysiol.90842.2008.
Dufner, D. a *et al.* (2005) 'Using 2H2O to study the influence of feeding on protein synthesis: effect of isotope equilibration in vivo vs. in cell culture.', *American journal of physiology. Endocrinology and metabolism*, 288(6), pp. E1277-83. doi: 10.1152/ajpendo.00580.2004.
Dufner, D. and Previs, S. F. (2003) 'Measuring in vivo metabolism using heavy water.', *Current opinion in clinical nutrition and metabolic care*, 6(5), pp. 511–7. doi: 10.1097/01.mco.0000087966.83880.73.
Edstrom, B. Y. L. *et al.* (1982) 'The contents of high-energy phosphates in different fibre types in skeletal muscles from rat, guinea-pig and man', *J Physiol*, 332, pp. 47–58.
Eisenberg, B. R. (1983) 'Quantitative ultrastructure of mammalian skeletal muscle', *Handbook of Physiology: Skeletal Muscle*, 10, pp. 73–112.

Evans, W. J. *et al.* (2019) 'D3-Creatine dilution and the importance of accuracy in the assessment of skeletal muscle mass', *Journal of Cachexia, Sarcopenia and Muscle*. doi: 10.1002/jcsm.12390.
Evans, W. J. and Lexell, J. (1995) 'Human Aging, Muscle Mass, and Fiber Type Composition', *The Journals of Gerontology: Series A*, 50A(Special_Issue), pp. 11–16. doi: 10.1093/gerona/50A.Special_Issue.11.
Fearon, K. C., Voss, A. C. and Hustead, D. S. (2006) 'Definition of cancer cachexia: effect of weight loss, reduced food intake, and systemic inflammation on functional status and prognosis.', *The American journal of clinical nutrition*, 83(6), pp. 1345–50.
Ferrando, A. A. *et al.* (2002) 'Testosterone administration to older men improves muscle function: Molecular and physiological mechanisms', *American Journal of Physiology - Endocrinology and Metabolism*, 282(3 45-3), pp. 601–607. doi: 10.1152/ajpendo.00362.2001.
Fielding, R. A. (1995) 'Effects of exercise training in the elderly: impact of progressive-resistance training on skeletal muscle and whole-body protein metabolism', *Proceedings of the Nutrition Society*, 54, pp. 665–675.
Fielding, R. a *et al.* (2011) 'Sarcopenia: an undiagnosed condition in older adults. Current consensus definition: prevalence, etiology, and consequences. International working group on sarcopenia.', *Journal of the American Medical Directors Association*. Elsevier Ltd, 12(4), pp. 249–56. doi: 10.1016/j.jamda.2011.01.003.
Fitch, C. D. and Sinton, D. W. (1964) *A Study of Creatine Metabolism in Diseases Causing Muscle Wasting **, *Journal of Clinical Investigation*.
Fitts, R. H., Riley, D. R. and Widrick, J. J. (2001) 'Functional and structural adaptations of skeletal muscle to microgravity', *Journal of Experimental Biology*, 204(18), pp. 3201–3208.
Forbes, G. B. and Reina, J. C. (1970) 'Adult lean body mass declines with age: Some longitudinal observations', *Metabolism*, 19(9), pp. 653–663. doi: 10.1016/0026-0495(70)90062-4.
Foster, G. L., Schoenheimer, R. and Rittenberg, D. (1938) 'Deuterium as an indicator in the study of intermediary metabolism: XIV. Biological Formation of Deuteroamino acids', *J. Biol. Chem*, 125, pp. 13–22.
Franchi, M. V. *et al.* (2015) 'Early structural remodeling and deuterium oxide-derived protein metabolic responses to eccentric and concentric loading in human skeletal muscle.', *Physiological reports*, 3(11), pp. e12593–e12593. doi: 10.14814/phy2.12593.
Franchi, M. V *et al.* (2014) 'Architectural, functional and molecular responses to concentric and eccentric loading in human skeletal muscle.', *Acta physiologica (Oxford, England)*, 210(3), pp. 642–54. doi: 10.1111/apha.12225.
Fricke, O. *et al.* (2010) 'Analyses of muscular mass and function: The impact on bone mineral density and peak muscle mass', *Pediatric Nephrology*, 25(12), pp. 2393–2400. doi: 10.1007/s00467-010-1517-y.
Frontera, W. R. *et al.* (2000) 'Aging of skeletal muscle: A 12-yr longitudinal study', *Journal of Applied Physiology*, 88(4), pp. 1321–

1326. doi: 10.1152/jappl.2000.88.4.1321.
Frontera, W. R. and Ochala, J. (2015) 'Skeletal Muscle: A Brief Review of Structure and Function', *Calcified Tissue International*, 96, pp. 183–195. doi: 10.1007/s00223-014-9915-y.
Fujita, S. *et al.* (2007) 'Basal muscle intracellular amino acid kinetics in women and men', *American Journal of Physiology - Endocrinology and Metabolism*, 292(1), pp. 1–19. doi: 10.1152/ajpendo.00173.2006.
Fujita, Satoshi *et al.* (2009) 'Regulation of Protein Metabolism in Exercise and Recovery Essential amino acid and carbohydrate ingestion before resistance exercise does not enhance postexercise muscle protein synthesis', 1, pp. 1730–1739. doi: 10.1152/japplphysiol.90395.2008.
Fulks, R. M., Li, J. B. and Goldberg, A. L. (1975) 'Effects of Insulin, Glucose, and Amino Acids on Protein Turnover in Rat Diaphragm', *Journal of Biological Chemistry*, 250(1), pp. 290–298.
Gallagher, D *et al.* (1997) 'Appendicular skeletal muscle mass: effects of age, gender, and ethnicity.', *Journal of applied physiology (Bethesda, Md.: 1985)*, 83(1), pp. 229–39.
Gallagher, Dympna *et al.* (1997) 'Appendicular skeletal muscle mass: Effects of age, gender, and ethnicity', *Journal of Applied Physiology*, 83(1), pp. 229–239. doi: 10.1152/jappl.1997.83.1.229.
Gallagher, I. J. *et al.* (2012) 'Suppression of skeletal muscle turnover in cancer cachexia: Evidence from the transcriptome in sequential human muscle biopsies', *Clinical Cancer Research*, 18(10), pp. 2817–2827. doi: 10.1158/1078-0432.CCR-11-2133.
Gardner, M. M. *et al.* (2001) 'Practical implementation of an exercise-based falls prevention programme.', *Age and ageing*, 30, pp. 77–83. doi: 10.1093/ageing/30.1.77.
Garlick, P. J. *et al.* (1994) 'Measurement of tissue protein synthesis rates in vivo: a critical analysis of contrasting methods.', *The American journal of physiology*, 266(3 Pt 1), pp. E287–E297.
Garlick, P. J., McNurlan, M. a. and Caso, G. (1997) 'Critical Assessment of Methods Used to Measure Protein Synthesis In Human Subjects', *The Yale journal of biology and medicine*, 70(1), pp. 65–76.
Gasier, H. G. *et al.* (2009) 'A comparison of 2H2O and phenylalanine flooding dose to investigate muscle protein synthesis with acute exercise in rats.', *American journal of physiology. Endocrinology and metabolism*, 297(1), pp. E252-9. doi: 10.1152/ajpendo.90872.2008.
Gasier, H. G., Fluckey, J. D. and Previs, S. F. (2010) 'The application of 2H2O to measure skeletal muscle protein synthesis.', *Nutrition & metabolism*, 7, p. 31. doi: 10.1186/1743-7075-7-31.
Gatt, I. *et al.* (2017) 'The Takei Handheld Dynamometer: An Effective Clinical Outcome Measure Tool for Hand and Wrist Function in Boxing', *Hand*. doi: 10.1177/1558944717707831.
Geva, T. (2006) 'Magnetic Resonance Imaging : Historical Perspective', *Journal of Cardiovascular Magnetic Resonance*, 8, pp. 573–580. doi: 10.1080/10976640600755302.
Gharahdaghi, N. *et al.* (2019) 'Testosterone therapy induces molecular programming augmenting physiological adaptations to

resistance exercise in older men', *Journal of Cachexia, Sarcopenia and Muscle*, pp. 1–19. doi: 10.1002/jcsm.12472.
Giampaoli, S. *et al.* (1999) 'Hand-grip strength predicts incident disability in non-disabled older men', *Age and Ageing*, 28(3), pp. 283–288. doi: 10.1093/ageing/28.3.283.
Gillies, A. R. and Lieber, R. L. (2011) 'Structure and Function of the Skeletal Muscle Extracellular Matrix', *Muscle Nerve*, 44(3), pp. 318–331. doi: 10.1002/mus.22094.Structure.
Gomes, M. D. *et al.* (2001) 'Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy', *Proceedings of the National Academy of Sciences of the United States of America*, 98(25), pp. 14440–14445. doi: 10.1073/pnas.251541198.
Goodpaster, B. H. *et al.* (2006) 'The loss of skeletal muscle strength, mass, and quality in older adults: the health, aging and body composition study.', *The journals of gerontology. Series A, Biological sciences and medical sciences*, 61(10), pp. 1059–64.
Gould, H. *et al.* (2014) 'Total and appendicular lean mass reference ranges for Australian men and women: The Geelong osteoporosis study', *Calcified Tissue International*, 94(4), pp. 363–372. doi: 10.1007/s00223-013-9830-7.
Green, J. and Britten, N. (1998) 'Qualitative research and evidence based medicine', *British Medical Journal*, 316(7139), pp. 1230–1232. doi: 10.1136/bmj.316.7139.1230.
Greenhaff, P. *et al.* (2019) 'Growing Older, Better Physiology's role in meeting the UK Government's healthy ageing mission', *Journal of Physiology*.
Greenhaff, P. L. *et al.* (1994) 'The metabolic responses of human type I and II muscle fibres during maximal treadmill sprinting.', *The Journal of Physiology*, 478(1), pp. 149–155. doi: 10.1113/jphysiol.1994.sp020238.
Gschwind, Y. J. *et al.* (2013) 'A best practice fall prevention exercise program to improve balance, strength / power, and psychosocial health in older adults: study protocol for a randomized controlled trial.', *BMC geriatrics*, 13(1), p. 105. doi: 10.1186/1471-2318-13-105.
Guglielmi, G. *et al.* (2016) 'The role of DXA in sarcopenia', *Aging Clinical and Experimental Research*. Springer International Publishing. doi: 10.1007/s40520-016-0589-3.
Guralnik, J. M. *et al.* (1994) 'A short physical performance battery assessing lower extremity function: association with self-reported disability and prediction of mortality and nursing home admission', *J Gerontol*, 49(2), pp. M85-94. doi: 10.1093/geronj/49.2.M85.
Gustafsson, T. *et al.* (2010) 'Effects of 3 days unloading on molecular regulators of muscle size in humans', *Journal of Applied Physiology*, 109(3), pp. 721–727. doi: 10.1152/japplphysiol.00110.2009.
Halket, J. M. *et al.* (2005) 'Chemical derivatization and mass spectral libraries in metabolic profiling by GC/MS and LC/MS/MS', *Journal of Experimental Botany*, 56(410), pp. 219–243. doi: 10.1093/jxb/eri069.
Harris, R. C., Söderlund, K. and Hultman, E. (1992) 'Elevation of creatine in resting and exercised muscle of normal subjects by

creatine supplementation.', *Clinical science (London, England : 1979)*, 83(3), pp. 367–74.
Hellmanns, K., McBean, K. and Thoirs, K. (2015) 'Magnetic Resonance Imaging in the measurement of whole body muscle mass: A comparison of interval gap methods', *Radiography*. Elsevier Ltd, 21(1), pp. e35–e39. doi: 10.1016/j.radi.2014.09.009.
Henderson, G. C. *et al.* (2009) 'Higher muscle protein synthesis in women than men across the lifespan, and failure of androgen administration to amend age-related decrements', *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2008/09/30. The Federation of American Societies for Experimental Biology, 23(2), pp. 631–641. doi: 10.1096/fj.08-117200.
Hershko, A. and Ciechanover, A. (1998) 'The ubiquitin system', *Annual review of biochemistry*, 67, pp. 425–479. doi: 10.1038/458421a.
Heymsfield, S. B. *et al.* (1990) 'Appendicular skeletal muscle mass: Measurement by dual- photon absorptiometry', *American Journal of Clinical Nutrition*, 52, pp. 214–218.
Heymsfield, S. B. *et al.* (2014) 'Assessing skeletal muscle mass: Historical overview and state of the art', *Journal of Cachexia, Sarcopenia and Muscle*, 5(1), pp. 9–18. doi: 10.1007/s13539-014-0130-5.
Heymsfield, S. B. *et al.* (2015) 'Skeletal muscle mass and quality: evolution of modern measurement concepts in the context of sarcopenia', *Proceedings of the Nutrition Society*, (December 2014), pp. 1–12. doi: 10.1017/S0029665115000129.
Hippisley-Cox, J., Coupland, C. and Brindle, P. (2017) 'Development and validation of QRISK3 risk prediction algorithms to estimate future risk of cardiovascular disease: Prospective cohort study', *BMJ (Online)*, 357(May), pp. 1–21. doi: 10.1136/bmj.j2099.
Ho-Pham, L. T., Nguyen, U. D. T. and Nguyen, T. V. (2014) 'Association between lean mass, fat mass, and bone mineral density: A meta-analysis', *Journal of Clinical Endocrinology and Metabolism*, 99(1), pp. 30–38. doi: 10.1210/jc.2013-3190.
Hoffmann, E. De (2005) 'Mass Spectrometry', *Kirk-Othmer Encyclopedia of Chemical Technology*, pp. 703–724. doi: 10.1002/0471238961.1301191913151.
Holm, S. (1979) 'A Simple Sequentially Rejective Multiple Test Procedure', *Scandinavian Journal of Statistics*, 6(2), pp. 65–70.
Hong, J. *et al.* (2016) 'Effects of home-based tele-exercise on sarcopenia among community-dwelling elderly adults: Body composition and functional fitness', *Experimental Gerontology*. Elsevier Inc., 87, pp. 33–39. doi: 10.1016/j.exger.2016.11.002.
Houmard, J. A., Smith, R. and Jendrasiak, G. L. (1995) 'Relationship between MRI relaxation time and muscle fiber composition', *Journal of Applied Physiology*, 78(3), pp. 807–809. doi: 10.1152/jappl.1995.78.3.807.
Houweling, M. *et al.* (2012) 'Technical note: quantification of plasma 1- and 3-methylhistidine in dairy cows by high-performance liquid chromatography-tandem mass spectrometry.', *Journal of dairy science*. Elsevier, 95(6), pp. 3125–30. doi: 10.3168/jds.2011-4769.

Hughes, V. a *et al.* (2002) 'Longitudinal changes in body composition in older men and women: role of body weight change and physical activity.', *The American journal of clinical nutrition*, 76(2), pp. 473–81.
Hunter, A. (1922) 'The Physiology of Creatine and Creatinine', *Physiol Rev*, 2, pp. 586–626.
Hunter, G. R., Mccarthy, J. P. and Bamman, M. M. (2004) 'Effects of Resistance Training on Older Adults', 34(5), pp. 329–348.
Hušek, P. (1998) 'Chloroformates in gas chromatography as general purposederivatizing agents', *Journal of Chromatography*, 717, pp. 57–91.
Huszar, G. *et al.* (1983) 'Protein-Bound and Soluble 3-Methylhistidine', *British Journal of Nutrition*, (49), pp. 287–294.
Ikai, M. and Fukunaga, T. (1970) 'A study on training effect on strength per unit cross-sectional area of muscle by means of ultrasonic measurement', *Internationale Zeitschrift für Angewandte Physiologie Einschließlich Arbeitsphysiologie*, 28(3), pp. 173–180. doi: 10.1007/BF00696025.
International Working Group on Sarcopenia (2011) 'Sarcopenia: An Undiagonosed Condition in Older Adults. Current Consensus Definition: Prevalence, Etiology, and Consequences', *J Am Med Dir Assoc.*, 12(4), pp. 249–256. doi: 10.1016/j.jamda.2011.01.003.Sarcopenia.
Ito, S. *et al.* (2015) 'Effectiveness of locomotion training in a home visit preventive care project: one-group pre-intervention versus post-intervention design study', *Journal of Orthopaedic Science*. Springer Japan, 20(6), pp. 1078–1084. doi: 10.1007/s00776-015-0760-2.
Jahn, L. A. *et al.* (1999) 'Tissue composition affects measures of postabsorptive human skeletal muscle metabolism: Comparison across genders', *Journal of Clinical Endocrinology and Metabolism*, 84(3), pp. 1007–1010. doi: 10.1210/jc.84.3.1007.
Janssen, I., Heymsfield, S. B. and Ross, R. (2002) 'Low Relative Skeletal Muscle Mass (Sarcopenia) in Older Persons Is Associated with Functional Impairment and Physical Disability', *Journal of American Geriatrics Society*, 50, pp. 889–896.
Jefferis, B. J. *et al.* (2014) 'Adherence to physical activity guidelines in older adults, using objectively measured physical activity in a population-based study', *BMC Public Health*, 14(1), p. 382. doi: 10.1186/1471-2458-14-382.
Jette, A. M. *et al.* (1996) 'A Home-based Exercise Program for Nondisabled Older Adults', *American Geriatrics Society*, 44(6), pp. 644–649.
Jette, A. M. *et al.* (1999) 'Exercise - It's never too late: The strong-for-life program', *American Journal of Public Health*, 89(1), pp. 66–72. doi: 10.2105/AJPH.89.1.66.
Joint FAO/WHO/UNU Expert Consultation, on Protein in Human Nutrition (2002 : Geneva Switzerland) Food Agriculture Organization of the United Nations, World Health Organisation, United Nations University, A. (2007) 'Protein and amino acid requirements in human nutrition : report of a joint FAO/WHO/UNU expert consultation'. World Health Organization (WHO technical report

series ; no. 935), pp. xi, 265 p.
Jones, P. J. and Leatherdale, S. T. (1991) 'Stable isotopes in clinical research: safety reaffirmed.', *Clin Sci (Lond).*, 80(4), pp. 277–80. doi: 10.1042/cs0800277.
Jones, S. E. *et al.* (2015) 'Sarcopenia in copd: Prevalence, clinical correlates and response to pulmonary rehabilitation', *Thorax*, 70(3), pp. 213–218. doi: 10.1136/thoraxjnl-2014-206440.
Katanik, J. *et al.* (2003) 'Measuring gluconeogenesis using a low dose of 2H2O : advantage of isotope fractionation during gas chromatography Measuring gluconeogenesis using a low dose of 2 H 2 O : advantage of isotope fractionation during gas chromatography', *American journal of physiology. Endocrinology and metabolism*, 284, pp. E1043–E1048. doi: 10.1152/ajpendo.00485.2002.
Katsanos, C. S. *et al.* (2006) 'A high proportion of leucine is required for optimal stimulation of the rate of muscle protein synthesis by essential amino acids in the elderly', 77550, pp. 381–387. doi: 10.1152/ajpendo.00488.2005.
Keller, K. and Engelhardt, M. (2013) 'Strength and muscle mass loss with aging process. Age and strength loss', *Muscles, Ligaments and Tendons Journal*, 3(4), pp. 346–350. doi: 10.11138/mltj/2013.3.4.346.
Kimball, S. R. (2013) 'Integration of signals generated by nutrients , hormones , and exercise in skeletal muscle', (C), pp. 4–9. doi: 10.3945/ajcn.113.068387.
Kinney, J. M. (1975) 'Metabolism of 3-Methylhistidine', 24(8), pp. 929–935.
Kjær, M. *et al.* (2006) 'Extracellular matrix adaptation of tendon and skeletal muscle to exercise', *J Anat.*, 208, pp. 445–450.
Koopman, R. (2011) 'Symposium 2 : Exercise and protein nutrition Dietary protein and exercise training in ageing Proceedings of the Nutrition Society', *Proceedings of the Nutrition Society*, 70, pp. 104–113. doi: 10.1017/S0029665110003927.
Kumar, V., Selby, A., *et al.* (2009) 'Age-related differences in the dose – response relationship of muscle protein synthesis to resistance exercise in young and old men', *Journal of Physiology*, 1, pp. 211–217. doi: 10.1113/jphysiol.2008.164483.
Kumar, V., Atherton, P., *et al.* (2009) 'Human muscle protein synthesis and breakdown during and after exercise', *Journal of Applied Physiology*, 106, pp. 2026–2039. doi: 10.1152/japplphysiol.91481.2008.
Kumar, V. *et al.* (2012) 'Muscle Protein Synthetic Responses to Exercise : Effects of Age , Volume , and Intensity', *J Gerontol A Biol Sci Med Sci*, 67(11), pp. 1170–1177. doi: 10.1093/gerona/gls141.
Kushmerick, M. J., Moerland, T. S. and Wiseman, R. W. (2006) 'Mammalian skeletal muscle fibers distinguished by contents of phosphocreatine, ATP, and Pi.', *Proceedings of the National Academy of Sciences*, 89(16), pp. 7521–7525. doi: 10.1073/pnas.89.16.7521.
Kyle, U. *et al.* (2001) 'Total Body Mass, Fat Mass, Fat-Free Mass, and Skeletal Muscle in Older People : Cross-Sectional Differences in 60-Year-Old Persons', *Journal of American Geriatrics Society*, 49,

pp. 1633–1640. doi: 10.1111/j.1532-5415.2001.49272.x.
Lang, C. H., Frost, R. A. and Vary, T. C. (2007) 'Regulation of muscle protein synthesis during sepsis and inflammation', 17033, pp. 453–459. doi: 10.1152/ajpendo.00204.2007.
Lehmann, W. D. (2016) 'A Timeline of Stable Isotopes and Mass Spectrometry in the Life Sciences', *Mass spectrometry reviews*, (DOI 10.1002/mas). doi: 10.1002/mas.
Leonard, M., Dunn, J. and Smith, G. (2014) 'A clinical biomarker assay for the quantification of d3-creatinine and creatinine using LC-MS/MS', *Bioanalysis*, 6(6), pp. 745–749. doi: 10.4155/bio.13.323.
Lexell, J., Taylor, C. C. and Sj, M. (1988) 'What is the cause of the ageing atrophy? Total number , size and proportion of different fiber types studied in whole vastus lateralis muscle from 15- to 83-year-old men', *Journal of the Neurological Sciences*, 84, pp. 275–294.
Liu, C.-J. and Latham, N. K. (2009) 'Progressive resistance strength training for improving physical function in older adults.', *The Cochrane database of systematic reviews*, 3(CD002759), pp. 1–227. doi: 10.1002/14651858.CD002759.pub2.
Long, C. L. *et al.* (1981) 'Urinary Excretion of 3-Methylhistidine: An Assessment of Muscle Protein Catabolism in Adult Normal Subjects and During Malnutrition, Sepsis, and Skeletal Trauma', *Metabolism*, 30(8), pp. 765–776.
Long, C. L. *et al.* (1988) 'Validity of 3-methylhistidine excretion as an indicator of skeletal muscle protein breakdown in humans', *Metabolism*, 37(9), pp. 844–849. doi: 10.1016/0026-0495(88)90118-7.
Lowry, S. F. *et al.* (1985) 'Whole-body protein breakdown and 3-methylhistidine excretion during brief fasting, starvation, and intravenous repletion in man.', *Annals of surgery*, 202(1), pp. 21–27. doi: 10.1097/00005373-198604000-00019.
Lu, X. *et al.* (2008) 'LC-MS-based metabonomics analysis', *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 866(1–2), pp. 64–76. doi: 10.1016/j.jchromb.2007.10.022.
Luger, E. *et al.* (2016) 'Effects of a Home-Based and Volunteer-Administered Physical Training, Nutritional, and Social Support Program on Malnutrition and Frailty in Older Persons: A Randomized Controlled Trial', *Journal of the American Medical Directors Association*. Elsevier Inc., 17(7), pp. 671.e9-671.e16. doi: 10.1016/j.jamda.2016.04.018.
Lukaski, H. C. (1998) 'Body Compostition in Exercise and Sport', in *Nutrition in Exercise and Sport*. 3rd Ed. New York: CRC Press, pp. 621–643.
Lum, J. J., DeBerardinis, R. J. and Thompson, C. B. (2005) 'Autophagy in metazoans: Cell survival in the land of plenty', *Nature Reviews Molecular Cell Biology*, 6(6), pp. 439–448. doi: 10.1038/nrm1660.
Lustgarten, M. S. and Fielding, R. A. (2011) 'Assessment of analytical methods used to measure changes in body composition in the elderly and recommendations for their use in phase II clinical trials', *Journal of Nutrition, Health and Aging*, 15(5), pp. 368–375. doi: 10.1007/s12603-011-0049-x.

Macdonald, A. J. *et al.* (2013) 'A novel oral tracer procedure for measurement of habitual myofibrillar protein synthesis', *Rapid Communications in Mass Spectrometry*, 27, pp. 1769–1777. doi: 10.1002/rcm.6622.
Maden-Wilkinson, T. M. *et al.* (2013) 'Comparison of MRI and DXA to measure muscle size and', *Journal of Musculoskeletal & Neuronal Interactions*, 13(3), pp. 282–290.
Mallet, A. I. and Down, S. (2009) *Dictionary of Mass Spectrometry*. Chichester: John Wiley & Sons, Ltd.
Malmstrom, T. K. and Morley, J. E. (2013) 'SARC-F: A simple questionnaire to rapidly diagnose sarcopenia', *Journal of the American Medical Directors Association*. American Medical Directors Association, Inc., 14(8), pp. 531–532. doi: 10.1016/j.jamda.2013.05.018.
Mammucari, C. *et al.* (2007) 'FoxO3 Controls Autophagy in Skeletal Muscle In Vivo', *Cell Metabolism*, 6(6), pp. 458–471. doi: 10.1016/j.cmet.2007.11.001.
Mangun, G. H. and Myers, V. C. (1939) 'Normal Creatine, Phosphorus and Potassium Content of Human Cardiac and Voluntary Muscle', *J Biol Chem*, 128, pp. 411–415.
Marcotte, G. R., West, D. W. D. and Baar, K. (2014) 'The Molecular Basis for Load-Induced Skeletal Muscle Hypertrophy.', *Calcified tissue international*. doi: 10.1007/s00223-014-9925-9.
Marcus, R. L. *et al.* (2010) 'SKELETAL MUSCLE FAT INFILTRATION : IMPACT OF AGE , INACTIVITY , AND EXERCISE', *Journal of Nutrition, Health and Aging*, 14(5), pp. 362–366.
Marshall, D., Johnell, O. and Wedel, H. (1996) 'Meta-analysis of how well measures of bone mineral density predict occurrence of osteoporotic fractures', *British Medical Journal*, 312(7041), pp. 1254–1259. doi: 10.1136/bmj.312.7041.1254.
Maruya, K. *et al.* (2016) 'Effect of a simple and adherent home exercise program on the physical function of community dwelling adults sixty years of age and older with pre-sarcopenia or sarcopenia.', *Journal of physical therapy science*, 28(11), pp. 3183–3188. doi: 10.1589/jpts.28.3183.
Masiero, E. *et al.* (2009) 'Autophagy Is Required to Maintain Muscle Mass', *Cell Metabolism*. Elsevier Ltd, 10(6), pp. 507–515. doi: 10.1016/j.cmet.2009.10.008.
Mayhew, A. J. *et al.* (2019) 'The prevalence of sarcopenia in community-dwelling older adults, an exploration of differences between studies and within definitions: A systematic review and meta-analyses', *Age and Ageing*, 48(1), pp. 48–56. doi: 10.1093/ageing/afy106.
Meier-Augenstein, W. (1999) 'Applied gas chromatography coupled to isotope ratio mass spectrometry', *Journal of Chromatography A*, 842(1–2), pp. 351–371. doi: 10.1016/S0021-9673(98)01057-7.
Meyer, R. A. and Prior, B. M. (1999) 'Functional Magnetic Resonance Imaging of Muscle', *Exercise and sport sciences reviews*, 28(2), pp. 89–92.
Miceli, A. (2012) 'Teoria Statistica Delle Classi e Calcolo Delle Probabilità', in Salkind, N. J. (ed.) *Encyclopedia of Research Design*. Thousand Oaks, pp. 1493–1494. doi:

10.4135/9781412961288.n455.
Minetto, M. A. *et al.* (2016) 'Ultrasound-Based Detection of Low Muscle Mass for Diagnosis of Sarcopenia in Older Adults', *PM and R*. American Academy of Physical Medicine and Rehabilitation, 8(5), pp. 453–462. doi: 10.1016/j.pmrj.2015.09.014.
Mitchell, W. K. *et al.* (2012) 'Sarcopenia , dynapenia , and the impact of advancing age on human skeletal muscle size and strength ; a quantitative review', *Frontiers in Physiology*, 3(July), pp. 1–18. doi: 10.3389/fphys.2012.00260.
Mitchell, W. K. *et al.* (2015) 'The impact of delivery profile of essential amino acids upon skeletal muscle protein synthesis in older men: clinical efficacy of pulse vs. bolus supply.', *American journal of physiology. Endocrinology and metabolism*, p. ajpendo.00112.2015. doi: 10.1152/ajpendo.00112.2015.
Mitsiopoulos, N. *et al.* (1998) 'Cadaver validation of skeletal muscle measurement by magnetic resonance imaging and computerized tomography Cadaver validation of skeletal muscle measurement by magnetic resonance imaging and computerized tomography', *Journal of Applied Physiology*, 85(1), pp. 115–122.
Mittendorfer, B. *et al.* (2005) 'Protein synthesis rates in human muscles: neither anatomical location nor fibre-type composition are major determinants.', *The Journal of physiology*, 563(Pt 1), pp. 203–211. doi: 10.1113/jphysiol.2004.077180.
Möller, P. and Brandt, R. (1982) 'The effect of physical training in elderly subjects with special reference to energy-rich phosphagens and myoglobin in leg skeletal muscle', *Clinical Physiology*, 2(4), pp. 307–314. doi: 10.1111/j.1475-097X.1982.tb00035.x.
Moore, D. R. *et al.* (2015) 'Protein ingestion to stimulate myofibrillar protein synthesis requires greater relative protein intakes in healthy older versus younger men', *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*, 70(1), pp. 57–62. doi: 10.1093/gerona/glu103.
Morley, J. E. *et al.* (2011) 'Sarcopenia With Limited Mobility: An International Consensus', *Journal of the American Medical Directors Association*, 12(6), pp. 403–409. doi: 10.1016/j.jamda.2011.04.014.
Morley, J. E. *et al.* (2014) 'Frailty, Sarcopenia and Diabetes', *Journal of the American Medical Directors Association*. AMDA – The Society for Post-Acute and Long-Term Care Medicine, 15(12), pp. 853–859. doi: 10.1016/j.jamda.2014.10.001.
Murphy, R. a *et al.* (2011) 'Influence of eicosapentaenoic acid supplementation on lean body mass in cancer cachexia', *British Journal of Cancer*. Nature Publishing Group, 105(10), pp. 1469–73. doi: 10.1038/bjc.2011.391.
Murton, A. J., Constantin, D. and Greenhaff, P. L. (2008) 'The involvement of the ubiquitin proteasome system in human skeletal muscle remodelling and atrophy', *Biochimica et Biophysica Acta - Molecular Basis of Disease*. Elsevier B.V., 1782(12), pp. 730–743. doi: 10.1016/j.bbadis.2008.10.011.
Narici, M. V and Maffulli, N. (2010) 'Sarcopenia : characteristics , mechanisms and functional significance', *British Medical Bulletin*, 95, pp. 139–159. doi: 10.1093/bmb/ldq008.

National Clinical Guideline Centre (2014) 'Chronic Kidney Disease (Partial Update) Early identification and management of chronic kidney disease in adults in primary and secondary care', *National Clinical Guideline Centre 2014*, (July), pp. 1–447. doi: 10.1016/j.clinthera.2017.01.002.
Nelson, M. E. *et al.* (2004) 'The Effects of Multidimensional Home-Based Exercise on Functional Performance in Elderly People', *Journal of Gerontology: Medical Sciences*, 59A(2), pp. 1–9. doi: 10.1016/j.aqpro.2013.07.003.
Office for National Statistics (2018) 'Population estimates for the UK, England and Wales, Scotland and Northern Ireland: mid-2017', *Office for National Statistics*, (July 2018), pp. 1–24. doi: 10.1088/0957-4484/19/9/095606.
Office of National Statistics (2019) *Living longer and old-age dependency – what does the future hold?*, *Office of National Statistics*. Available at: https://www.ons.gov.uk/peoplepopulationandcommunity/birthsdeathsandmarriages/ageing/articles/livinglongerandoldagedependencywhatdoesthefuturehold/2019-06-24.
Oshima, T. and Tamiya, N. (1961) 'Mechanism of Transaminase Action', *Biochemical Journal*, 78, pp. 116–119.
Paddon-Jones, D. *et al.* (2004) 'Amino acid ingestion improves muscle protein synthesis in the young and elderly.', *American journal of physiology. Endocrinology and metabolism*, 286(3), pp. E321-8. doi: 10.1152/ajpendo.00368.2003.
Park, S. W. *et al.* (2007) 'Accelerated Loss of Skeletal Muscle Strength in Older Adults with Type 2 Diabetes', *Diabetes Care*, 30, pp. 1507–1512. doi: 10.2337/dc06-2537.Additional.
Peterson, M. D. and Gordon, P. M. (2011) 'Resistance exercise for the aging adult: Clinical implications and prescription guidelines', *American Journal of Medicine*. Elsevier Inc., 124(3), pp. 194–198. doi: 10.1016/j.amjmed.2010.08.020.
Phillips, B. *et al.* (2012) 'Resistance exercise training improves age-related declines in leg vascular conductance and rejuvenates acute leg blood flow responses to feeding and exercise.', *Journal of applied physiology*, 112, pp. 347–53. doi: 10.1152/japplphysiol.01031.2011.
Phillips, B. *et al.* (2013) 'Resistance exercise training improves age-related declines in leg vascular conductance and rejuvenates acute leg blood flow responses to feeding and exercise Resistance exercise training improves age-related declines in leg vascular conductance and rejuve'. doi: 10.1152/japplphysiol.01031.2011.
Phillips, B. E. *et al.* (2015) 'The effects of resistance exercise training upon macro and micro-circulatory responses to feeding and skeletal muscle protein anabolism, in older men', *The Journal of Physiology*, 63(5), p. n/a-n/a. doi: 10.1113/JP270343.
Phillips, S. M. *et al.* (1997) 'Mixed muscle protein synthesis and breakdown after resistance exercise in humans.', *The American journal of physiology*, 273, pp. E99–E107.
Phillips, S. M. *et al.* (1999) 'Resistance training reduces the acute exercise-induced increase in muscle protein turnover.', *The American journal of physiology*, 276(1 Pt 1), pp. E118-24.

Phillips, S M, Glover, E. I. and Rennie, M. J. (2009) 'Alterations of protein turnover underlying disuse atrophy in human skeletal muscle', *Journal of Applied Physiology*, 107, pp. 645–654. doi: 10.1152/japplphysiol.00452.2009.
Phillips, S. M., Glover, E. I. and Rennie, M. J. (2009) 'Alterations of protein turnover underlying disuse atrophy in human skeletal muscle', *Journal of Applied Physiology*, 107(3), pp. 645–654. doi: 10.1152/japplphysiol.00452.2009.
Płotka-Wasylka, J. M. *et al.* (2015) 'Chemical Derivatization Processes Applied to Amine Determination in Samples of Different Matrix Composition', *Chemical Reviews*, p. 150529125335004. doi: 10.1021/cr4006999.
Plotnikoff, R. C. *et al.* (2010) 'Multicomponent, home-based resistance training for obese adults with type 2 diabetes: a randomized controlled trial.', *International journal of obesity (2005)*. Nature Publishing Group, 34(12), pp. 1733–41. doi: 10.1038/ijo.2010.109.
Ponti, F. *et al.* (2020) 'Aging and Imaging Assessment of Body Composition : From Fat to Facts', 10(January). doi: 10.3389/fendo.2019.00861.
Previs, S. F. *et al.* (2004) 'Quantifying rates of protein synthesis in humans by use of 2H2O: application to patients with end-stage renal disease.', *American journal of physiology. Endocrinology and metabolism*, 286(4), pp. E665-72. doi: 10.1152/ajpendo.00271.2003.
Previs, S. F. *et al.* (2011) 'Quantifying cholesterol synthesis in vivo using 2 H 2 O : enabling back-to-back studies in the same subject', 52. doi: 10.1194/jlr.D014993.
Proctor, D. N. *et al.* (1999) 'Comparison of techniques to estimate total body skeletal muscle mass in people of different age groups', *American Journal of Physiology - Endocrinology and Metabolism*, 277(3 40-3). doi: 10.1152/ajpendo.1999.277.3.e489.
Prokůpek, J. *et al.* (2014) 'Development and first experimental tests of Faraday cup arrayc', *Review of Scientific Instruments*, 85(1). doi: 10.1063/1.4859496.
Proud, C. G. (2007) 'Signalling to translation: how signal transduction pathways control the protein synthetic machinery.', *The Biochemical journal*, 403(2), pp. 217–34. doi: 10.1042/BJ20070024.
Proud, C. G. (2009) 'mTORC1 signalling and mRNA translation.', *Biochemical Society transactions*, 37(Pt 1), pp. 227–231. doi: 10.1042/BST0370227.
Puthucheary, Z. A. *et al.* (2013) 'Acute Skeletal Muscle Wasting in Critical Illness', pp. 1–10. doi: 10.1001/jama.2013.278481.
Reed, R. L. *et al.* (1991) 'The Relationship between Muscle Mass and Muscle Strength in the Elderly', *Journal of the American Geriatrics Society*, 39(6), pp. 555–561. doi: 10.1111/j.1532-5415.1991.tb03592.x.
Reeves, N. D., Maganaris, C. N. and Narici, M. V. (2004) 'Ultrasonographic assessment of human skeletal muscle size', *European Journal of Applied Physiology*, 91(1), pp. 116–118. doi: 10.1007/s00421-003-0961-9.

Reiss, J. *et al.* (2019) 'Consequences of applying the new EWGSOP2 guideline instead of the former EWGSOP guideline for sarcopenia case finding in older patients', *Age and Ageing*, pp. 1–6. doi: 10.1093/ageing/afz035.
Relaix, F. and Zammit, P. S. (2012) 'Satellite cells are essential for skeletal muscle regeneration : the cell on the edge returns centre stage', *Development*, 139, pp. 2845–2856. doi: 10.1242/dev.069088.
Rennie, M. J. *et al.* (1982) 'Muscle protein synthesis measured by stable isotope techniques in man : the effects of feeding and fasting', *Clinical Science*, (63), pp. 519–523.
Rennie, M. J. (1985) 'Muscle protein turnover and the wasting due to injury and disease.', *British medical bulletin*, 41(3), pp. 257–264.
Rennie, M. J. (1999) 'An introduction to the use of tracers in nutrition and metabolism', *Proceedings of the Nutrition Society*, 58, pp. 935–944. doi: 10.1017/S002966519900124X.
Rennie, M. J. *et al.* (2006) 'Branched-chain amino acids as fuels and anabolic signals in human muscle.', *The Journal of nutrition*, 136, pp. 264S–8S. doi: 136/1/264S [pii].
Rennie, M., Smith, K. and Watt, P. (1994) 'Measurement of human tissue protein synthesis: an optimal approach', *The American journal of physiology*, 266(29), pp. 298–307.
Ribeiro, F. *et al.* (2009) 'Impact of low cost strength training of dorsi- and plantar flexors on balance and functional mobility in institutionalized elderly people', *Geriatrics and Gerontology International*, 9(1), pp. 75–80. doi: 10.1111/j.1447-0594.2008.00500.x.
Riggs, B. L. *et al.* (1981) 'Differential changes in bone mineral density of the appendicular and axial skeleton with aging. Relationship to spinal osteoporosis', *Journal of Clinical Investigation*, 67(2), pp. 328–335. doi: 10.1172/JCI110039.
Rittenberg, D. *et al.* (1938) 'Deuterium as an indicator in the study of intermediary metabolism: XIII. The stability of Hydrogen in amino acids', *Journal of Biological Chemistry*.
Rittenberg, D. and Schoenheimer, R. (1937a) 'Deuterium as an indicator in the study of intermediary metabolism: VIII. Hydrogenation of fatty acids in the animal organism', *Journal of Biological Chemistry*, 117, pp. 485–490.
Rittenberg, D. and Schoenheimer, R. (1937b) 'Deuterium as an indicator in the study of intermediary metabolism: XI. Further studies on the biological uptake of deuterium into organic substances, with special reference to fat and cholesterol formation', *Journal of Biological Chemistry*, 121, pp. 235–253.
Rooyackers, O. E. and Nair, K. S. (1997) 'Hormonal regulation of human muscle protein metabolism.', *Annual review of nutrition*, 17, pp. 457–485. doi: 10.1146/annurev.nutr.17.1.457.
Rosenberg, I. H. (1989) 'Summary Comments', *The American journal of clinical nutrition*, 50, pp. 1231–1233.
Rosenberg, I. H. (1997) 'Sarcopenia: Origins and Clinical Relevance', *American Society for Nutritional Sciences*, p. 3166.
Ross, R. *et al.* (1996) 'Influence of diet and exercise on skeletal muscle and visceral adipose tissue in men.', *Journal of applied*

physiology (Bethesda, Md. : 1985), 81(6), pp. 2445–2455.
Rudrappa, S. S. *et al.* (2016) 'Human Skeletal Muscle Disuse Atrophy: Effects on Muscle Protein Synthesis, Breakdown, and Insulin Resistance—A Qualitative Review', *Frontiers in Physiology*, 7(August), pp. 1–10. doi: 10.3389/fphys.2016.00361.
Sanada, K. *et al.* (2006) 'Prediction and validation of total and regional skeletal muscle mass by ultrasound in Japanese adults', *European Journal of Applied Physiology*, 96(1), pp. 24–31. doi: 10.1007/s00421-005-0061-0.
Sandri, M. (2010) 'Autophagy in skeletal muscle', *FEBS Letters*. Federation of European Biochemical Societies, 584(7), pp. 1411–1416. doi: 10.1016/j.febslet.2010.01.056.
Sant'Ana Pereira, J. A. A. *et al.* (1996) 'Myosin heavy chain isoform expression and high energy phosphate content in human muscle fibres at rest and post-exercise', *Journal of Physiology*, 496(2), pp. 583–588. doi: 10.1113/jphysiol.1996.sp021709.
Schmalbruch, H. and Lewis, D. M. (2000) 'Dynamics of nuclei of muscle fibers and connective tissue cells in normal and denervated rat muscles', *Muscle and Nerve*, 23(4), pp. 617–626. doi: 10.1002/(SICI)1097-4598(200004)23:4<617::AID-MUS22>3.0.CO;2-Y.
Schoenheimer, R. and Rittenberg, D. (1936) 'Deuterium As An Indicator In the Study Of Intermediary Meatbolism VI. Synthesis and Destruction of Fatty Acids in The Organism', *Journal of Biological Chemistry*.
Schoenheimer, R., Rittenberg, D. and Graff, M. (1935) 'Deuterium as an indicator in the study of intermediary metabolism:IV. The mechanism of coprosterol formation', *Journal of Biological Chemistry*, pp. 183–192.
Scott, D. *et al.* (2014) 'Operational definitions of sarcopenia and their associations with 5-year changes in falls risk in community-dwelling middle-aged and older adults', *Osteoporosis International*, 25(1), pp. 187–193. doi: 10.1007/s00198-013-2431-5.
Scott, W., Stevens, J. and Binder-macleod, S. A. (2001) 'Human Skeletal Muscle Fiber Type Classifications', *Physical Therapy*, 81(11), pp. 1810–1816. doi: 10.1093/ptj/81.11.1810.
Sessions, A. L. *et al.* (1999) 'Fractionation of hydrogen isotopes in lipid biosynthesis', *Organic Geochemistry*, 30(9), pp. 1193–1200.
Sessions, A. L. (2006) 'Review Isotope-ratio detection for gas chromatography', *J Sep Sci*, 29, pp. 1946–1961. doi: 10.1002/jssc.200600002.
Sessions, A. L., Burgoyne, T. W. and Hayes, J. M. (2001) 'Determination of the H3 factor in hydrogen isotope ratio monitoring mass spectrometry', *Analytical Chemistry*, 73(2), pp. 200–207. doi: 10.1021/ac000488m.
Severinsen, K. *et al.* (2007) 'Atrophy of Foot Muscles in Diabetic Patients Can Be Detected with Ultrasonography', *Diabetes Care*, 30(12), pp. 3053–3057. doi: 10.2337/dc07-0108.Additional.
Shankaran, M. *et al.* (2018) 'Dilution of oral D 3 -Creatine to measure creatine pool size and estimate skeletal muscle mass: development of a correction algorithm', *Journal of Cachexia, Sarcopenia and Muscle*. doi: 10.1002/jcsm.12278.

Sheffield-Moore, M. *et al.* (2014) 'Isotopic decay of urinary or plasma 3-methylhistidine as a potential biomarker of pathologic skeletal muscle loss', *Journal of Cachexia, Sarcopenia and Muscle*, 5(1), pp. 19–25. doi: 10.1007/s13539-013-0117-7.
Sheikh, R. A. *et al.* (1999) 'AIDS-related myopathy', pp. 79–86.
Shen, W. *et al.* (2004) 'Total body skeletal muscle and adipose tissue volumes: estimation from a single abdominal cross-sectional image.', *Journal of applied physiology (Bethesda, Md. : 1985)*, 97(6), pp. 2333–8. doi: 10.1152/japplphysiol.00744.2004.
Silveira, P. *et al.* (2013) 'Tablet-Based Strength-Balance Training to Motivate and Improve Adherence to Exercise in Independently Living Older People: A Phase II Preclinical Exploratory Trial.', *Journal of Medical Internet Research*, 15(8), pp. e159–e159. doi: 10.2196/jmir.2579.
Smith, G. I. *et al.* (2008) 'Differences in Muscle Protein Synthesis and Anabolic Signaling in the Postabsorptive State and in Response to Food in 65 – 80 Year Old Men and Women', *PloS one*, 3(3), pp. 1–9. doi: 10.1371/journal.pone.0001875.
Smith, G. I. *et al.* (2009) 'No major sex differences in muscle protein synthesis rates in the postabsorptive state and during hyperinsulinemia-hyperaminoacidemia in middle-aged adults', *Journal of Applied Physiology*, 107(4), pp. 1308–1315. doi: 10.1152/japplphysiol.00348.2009.
Smith, G. I. *et al.* (2010) 'Timing of the initial muscle biopsy does not affect the measured muscle protein fractional synthesis rate during basal, postabsorptive conditions.', *Journal of applied physiology (Bethesda, Md. : 1985)*, 108(2), pp. 363–368. doi: 10.1152/japplphysiol.00957.2009.
Smith, G. I. *et al.* (2012) 'Sexually dimorphic effect of aging on skeletal muscle protein synthesis', *Biology of Sex Differences*. Biology of Sex Differences, 3(1), p. 1. doi: 10.1186/2042-6410-3-11.
Smith, G. I. *et al.* (2013) 'Human muscle protein turnover −− why is it so variable ? Human muscle protein turnover — why is it so variable ?', *J*, (November 2010), pp. 480–491. doi: 10.1152/japplphysiol.00125.2010.
Smith, G. I., Patterson, B. W. and Mittendorfer, B. (2010) 'Human muscle protein turnover −− why is it so variable ?', *Journal of Applied Physiology*, 110, pp. 480–491. doi: 10.1152/japplphysiol.00125.2010.
Smith, K. *et al.* (1992) 'Flooding with L-[1-13C]leucine stimulates human muscle protein incorporation of continuously infused L-[1-13C]valine.', *The American journal of physiology*, 262(3 Pt 1), pp. E372–E376.
Smith, K. *et al.* (1998) 'Effects of flooding amino acids on incorporation of labeled amino acids into human muscle protein.', *The American journal of physiology*, 275(1 Pt 1), pp. E73-8.
Snow-Harter, C. *et al.* (1990) 'Muscle strength as a predictor of bone mineral density in elderly women', *Journal of Bone and Mineral Research*, 5(6), pp. 589–595.
Snow-Harter, C. *et al.* (1992) 'Bone mineral density, muscle strength, and recreational exercise in men', *Journal of Bone and*

Mineral Research, 7(11), pp. 1291–1296. doi: 10.1002/jbmr.5650071108.
Stephens, N. A. *et al.* (2010) 'Using transcriptomics to identify and validate novel biomarkers of human skeletal muscle cancer cachexia', *Genome Medicine*, 2(1), pp. 1–12. doi: 10.1186/gm122.
Stevens, J. R., Masud, A. Al and Suyundikov, A. (2017) 'A comparison of multiple testing adjustment methods with block-correlation positivelydependent tests', *PLoS ONE*, 12(4), pp. 1–12. doi: 10.1371/journal.pone.0176124.
Stevens, P. E. *et al.* (2007) 'Chronic kidney disease management in the United Kingdom: NEOERICA project results', *Kidney International*. Elsevier Masson SAS, 72(1), pp. 92–99. doi: 10.1038/sj.ki.5002273.
Stimpson, S. a. *et al.* (2012) 'Total-body creatine pool size and skeletal muscle mass determination by creatine-(methyl-d3) dilution in rats', *Journal of Applied Physiology*, 112(17), pp. 1940–1948. doi: 10.1152/japplphysiol.00122.2012.
Studenski, S. A. *et al.* (2014) 'The FNIH sarcopenia project: Rationale, study description, conference recommendations, and final estimates', *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*, 69 A(5), pp. 547–558. doi: 10.1093/gerona/glu010.
Symons, T. B. *et al.* (2009) 'Artificial gravity maintains skeletal muscle protein synthesis during 21 days of simulated microgravity', *Journal of Applied Physiology*, 107(1), pp. 34–38. doi: 10.1152/japplphysiol.91137.2008.
Taekema, D. G. *et al.* (2010) 'Handgrip strength as a predictor of functional, psychological and social health. A prospective population-based study among the oldest old', *Age and Ageing*, 39(3), pp. 331–337. doi: 10.1093/ageing/afq022.
Tallan, H. H., Stein, W. H. and Moore, S. (1954) '3-Methylhistidine, A New Amino Acid From Human Urine', *Journal of Biological Chemistry*, 206(2), pp. 825–34.
Tarantino, U. *et al.* (2015) 'Sarcopenia and fragility fractures: Molecular and clinical evidence of the bone-muscle interaction', *Journal of Bone and Joint Surgery - American Volume*, 97(5), pp. 429–437. doi: 10.2106/JBJS.N.00648.
Tarnopolsky, M. A., MacDougall, J. D. and Atkinson, S. A. (1988) 'Influence of protein intake and training status on nitrogen balance and lean body mass.', *Journal of applied physiology (Bethesda, Md. : 1985)*, 64(1), pp. 187–93. doi: 10.1152/jappl.1988.64.1.187.
Thiebaud, R. S., Funk, M. D. and Abe, T. (2014) 'Home-based resistance training for older adults: a systematic review.', *Geriatrics & gerontology international*, 14(4), pp. 750–7. doi: 10.1111/ggi.12326.
Thomas, E. L. *et al.* (1998) 'Magnetic resonance imaging of total body fat', *Journal of Applied Physiology*, 85(5), pp. 1778–1785. doi: 10.1152/jappl.1998.85.5.1778.
Tieland, M., Trouwborst, I. and Clark, B. C. (2017) 'Skeletal muscle performance and ageing', *Journal of Cachexia, Sarcopenia and Muscle*, (November 2017), pp. 3–19. doi: 10.1002/jcsm.12238.
Tipton, K. D., Hamilton, D. L. and Gallagher, I. J. (2018) 'Assessing

the Role of Muscle Protein Breakdown in Response to Nutrition and Exercise in Humans', *Sports Medicine*. Springer International Publishing, 48(s1), pp. 53–64. doi: 10.1007/s40279-017-0845-5.
Tisdale, M. J. (2009) 'Mechanisms of Cancer Cachexia', *Physiological reviews*, 89, pp. 381–410. doi: 10.1152/physrev.00016.2008.
Trost, S. G. *et al.* (2002) 'Correlates of adults' participation in physical activity: review and update.', *Medicine & Science in Sports & Exercise*, 34(12), pp. 1996–2001. doi: 10.1249/01.MSS.0000038974.76900.92.
Urso, M. L. *et al.* (2006) 'Analysis of human skeletal muscle after 48 h immobilization reveals alterations in mRNA and protein for extracellular matrix components', *Journal of Applied Physiology*, 101(4), pp. 1136–1148. doi: 10.1152/japplphysiol.00180.2006.
Ussing, H. (1941) 'The rate of protein renewal in mice and rats studied by means of heavy hydrogen', *Acta Physiologica Scan.*, pp. 209–222.
Vaughan, V. C., Martin, P. and Lewandowski, P. A. (2013) 'Cancer cachexia : impact , mechanisms and emerging treatments', pp. 95–109. doi: 10.1007/s13539-012-0087-1.
Vestal, M. L. (1984) 'High-Performance Liquid Spectrometry', *Science*, 226(4672), pp. 275–281.
Villaseñor, A. *et al.* (2012) 'Prevalence and prognostic effect of sarcopenia in breast cancer survivors: The HEAL Study', *Journal of Cancer Survivorship*, 6(4), pp. 398–406. doi: 10.1007/s11764-012-0234-x.
Visser, M. *et al.* (1999) 'Validity of fan-beam dual-energy X-ray absorptiometry for measuring fat-free mass and leg muscle mass. Health, Aging, and Body Composition Study--Dual-Energy X-ray Absorptiometry and Body Composition Working Group.', *Journal of Applied Physiology*, 87(4), pp. 1513–1520.
Visser, M. *et al.* (2005) 'Muscle Mass , Muscle Strength , and Muscle Fat Infiltration as Predictors of Incident Mobility Limitations in Well-Functioning Older Persons', 60(3), pp. 324–333.
Volpi, E. *et al.* (2001) 'Basal Muscle Amino Acid Kinetics and Protein Synthesis in Healthy Young and Older Men', *JAMA*, 286(10), pp. 1206–1212. doi: 10.1016/j.biotechadv.2011.08.021.Secreted.
Wang, Z. *et al.* (2003) 'Whole-body skeletal muscle mass: Development and validation of total-body potassium prediction models1-3', *American Journal of Clinical Nutrition*, 77(1), pp. 76–82.
Waterlow, J. C. (1995) 'Whole-Body Protein Turnover in Humans--Past, Present, and Future', *Annual review of nutrition*, (15), pp. 57–92.
Watt, P. W. *et al.* (1991) 'Isolation of aminoacyl-tRNA and its labeling with stable-isotope tracers : Use in studies of human tissue protein synthesis', *Proc. Natl. Acad. Sci USA*, 88(July), pp. 5892–5896.
Weber, M. A. *et al.* (2009) 'Morphology, metabolism, microcirculation, and strength of skeletal muscles in cancer-related cachexia', *Acta Oncologica*, 48(1), pp. 116–124. doi: 10.1080/02841860802130001.
Welch, E., Wang, W. and Peltz, S. (2000) '11 Translation Termination: It's Not the End of the Story', *Cold Spring Harbor*

Monograph Archive, 39, pp. 467–485. doi: 10.1080/01956051.2010.508504.
Wilkinson, D. J. *et al.* (2014) 'A validation of the application of D2O stable isotope tracer techniques for monitoring day-to-day changes in muscle protein subfraction synthesis in humans.', *American journal of physiology. Endocrinology and metabolism*, 306(5), pp. E571-9. doi: 10.1152/ajpendo.00650.2013.
Wilkinson, D. J. *et al.* (2015) 'Internal comparison between deuterium oxide (D_2O) and L - [*ring* - $^{13}C_6$] phenylalanine for acute measurement of muscle protein synthesis in humans', *Physiological Reports*, 3(7), p. e12433. doi: 10.14814/phy2.12433.
Wilkinson, D. J. (2016) 'Historical and Contemporary Stable Isotope Tracer Approaches To Studying Mammalian Protein Metabolism', *Mass Spectrometry*, (10.1002/mas). doi: 10.1002/mas.
Wilkinson, D. J. *et al.* (2017) 'Effects of leucine-enriched essential amino acid and whey protein bolus dosing upon skeletal muscle protein synthesis at rest and after exercise in older women', *Clinical Nutrition*. Elsevier Ltd, (September), pp. 1–12. doi: 10.1016/j.clnu.2017.09.008.
Willer, B. *et al.* (2000) 'Effects of creatine supplementation on muscle weakness in patients with rheumatoid arthritis', *Rheumatology*, 39(3), pp. 293–298. doi: 10.1093/rheumatology/39.3.293.
Wolfe, R. R. and Chinkes, D. L. (2005) *Isotope Tracers in Metabolic Research: Principles and Practice of Kinetic Analysis*. 2nd edn. New Jersey: John Wiley & Sons.
Wyss, M and Kaddurah-Daouk, R. (2000) 'Creatine and creatinine metabolism.', *Physiological reviews*, 80(3), pp. 1107–213.
Wyss, Markus and Kaddurah-Daouk, R. (2000) 'Creatine and Creatinine Metabolism', *Physiological Reviews*, 80(3), pp. 1107–1213.
Yamauchi, J., Nakayama, S. and Ishii, N. (2009) 'Effects of bodyweight-based exercise training on muscle functions of leg multi-joint movement in elderly individuals', *Geriatrics and Gerontology International*, 9(3), pp. 262–269. doi: 10.1111/j.1447-0594.2009.00530.x.
Yarasheski, K. E. *et al.* (1992) 'Measurement of Muscle Protein Fractional Synthetic Rate by Capillary Gas Chromatography/Combustion Isotope Ratio Mass Spectrometry', *Biol Mass Spectrom.*, 21(10), pp. 486–490. doi: 10.1037/a0013262.Open.
Yates, J. R., Ruse, C. I. and Nakorchevsky, A. (2009) 'Proteomics by mass spectrometry: approaches, advances, and applications.', *Annual review of biomedical engineering*, 11, pp. 49–79. doi: 10.1146/annurev-bioeng-061008-124934.
Yoav, B. and Hochberg, Y. (1995) 'Controlling the False Discovery Rate : A Practical and Powerful Approach to Multiple Testing Author', *Journal of the Royal Statistical Society. Series B (Methodological)*, 57(1), pp. 289–300.
Young, V. R. V. *et al.* (1972) 'Metabolism of administered 3-methylhistidine', *journal of Biological Chemistry*, 247(14), pp. 3592–3600.

Zhang, X. *et al.* (1996) 'An isotopic method for measurement of muscle protein fractional breakdown rate in vivo', *American Journal of Physiology- Endocrinology and Metabolism*, 270(33), pp. E759-67.
Zhang, Y. *et al.* (2012) 'Calibration and data processing in gas chromatography combustion isotope ratio mass spectrometry', *Drug Testing and Analysis*, 4(12), pp. 912–922. doi: 10.1002/dta.394.
Zierath, J. R. and Hawley, J. a (2004) 'Skeletal muscle fiber type: influence on contractile and metabolic properties.', *PLoS biology*, 2(10), p. e348. doi: 10.1371/journal.pbio.0020348.
Zion, A. S. *et al.* (2003) 'A home-based resistance-training program using elastic bands for elderly patients with orthostatic hypotension', *Clinical Autonomic Research*, 13(4), pp. 286–292. doi: 10.1007/s10286-003-0117-3.
Zlot, A. I. *et al.* (2006) 'Environmental, Transportation, Social, and Time Barriers to Physical Activity', *Journal of Physical Activity and Health*, 3, pp. 15–21.