Uncovering the interaction between bone and muscle in older adults: effects of exercise

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

by

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Bachelor of Exercise and Sports Science

Graduate Diploma in Exercise and Rehabilitation

Master of Clinical Exercise Science and Rehabilitation

2022

Institute for Health and Sport (IHES)

Victoria University

Melbourne, Australia

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Abstract

Ageing is characterised by a simultaneous loss of muscle mass, strength and physical performance (sarcopenia) and bone mass (osteoporosis). These changes in muscle and bone can lead to reduced physical function, increased falls and fractures and a poorer quality of life. Due to the ageing population and increases in sedentary behaviours, sarcopenia and osteoporosis prevalence and associated burdens are predicted to rise. The simultaneous loss of muscle and bone raised the hypothesis that bone and muscle are not only linked anatomically, but also metabolically and chemically. However, it is still not clear how bone-derived factors are involved in this crosstalk.

Bone is an endocrine organ, releasing hormones affecting distant tissues and organs. Osteocalcin (OC) is the most abundant non-collagenous protein in bone. Its total serum levels (tOC) are used clinically as a bone turnover marker (BTM). The undercarboxylated form of OC (ucOC) is considered bio-active, involved in energy metabolism and possibly muscle mass maintenance and strength, at least in rodents. Evidence from human studies is limited and contradictory, in part because most research has focused on tOC, rather than the ucOC.

Exercise improves muscle and bone mass, as well as muscle strength, while inactivity has deleterious effects on both organs. Consequently, exercise is a cornerstone approach to maintain and preserve musculoskeletal health in adults, and can be used as a tool to investigate bone-muscle crosstalk. The primary aim of this PhD thesis was to identify the normal range of ucOC across the adult human lifespan, and to explore whether ucOC and other BTMs are related to muscle mass, strength and physical performance. I also explored whether acute exercise can affect the relationships between ucOC and muscle function (strength and physical performance) in older adults. The specific aims were:

Study 1: to develop age-based reference ranges for OC and its forms and ratios in healthy adult men. Overall, 236 adult men participated in the study (18 to 92 years old). Serum samples were analysed for tOC and ucOC (using the hydroxyapatite binding method) and carboxylated OC (cOC), ucOC/tOC and cOC/tOC ratios were calculated. Ageing was associated with a “U” shaped pattern for tOC, cOC and ucOC levels. The ucOC/tOC ratio was higher, while cOC/tOC ratio was lower, in men of advanced age, demonstrating that OC ratios may be better measures than the absolute values to identify age-related changes in OC.

Study 2: to test the hypothesis that the serum ucOC absolute value and ucOC/tOC ratio are associated with muscle function and long-term risk for falls-related hospitalisations using a large longitudinal dataset (15 years) in older women (n=1261, mean age 75.2±2.7 years). In older women, a higher ucOC/tOC ratio was related to poorer physical function, including the long-term decline in physical function and increased risk of falls-related hospitalisations. Early The identification of women at higher risk for functional decline using the ucOC/tOC ratio may enable prevention and intervention strategies to occur early, reducing future risk for injurious falls.

Study 3: to perform a systematic review to examine the effects of acute exercise on BTMs in adults over the age of 50 years and identify whether BTM responses are determined by exercise mode, intensity, age and sex. Thirteen studies were included: eight in middle-aged adults (n= 275, 212 women/63 men, mean age= 57.9±1.5 years) and five in older adults (n= 93, 50 women/43 men, mean age= 68.2±2.2 years). Eleven studies included aerobic exercise (AE) (7 middle-aged/4 older adults) and two included resistance exercise (RE) (both in middle-aged adults). AE increased C-terminal telopeptide of type I collagen (CTX), alkaline phosphatase (ALP) and bone-ALP in middle-aged and older adults. AE also increased tOC in middle-aged men, and procollagen I carboxyterminal propeptide (PICP) and cross-linked carboxyterminal telopeptide of type I collagen (ICTP) in older women. In middle-aged adults, RE combined with impact exercise (jumping) had no effect on tOC or BALP, but led to a decrease in CTX. Jumping alone increased P1NP and tOC in middle-aged women. Acute exercise is an effective tool to modify BTMs, but the response appears to be specific to exercise modality, intensity, age and sex.

Study 4: to test the hypotheses that **a)** at baseline, serum ucOC and other BTMs are associated with muscle function, **b)** acute exercise can alter ucOC and BTMs and **c)** muscle function at baseline is related to the acute exercise responses of these biomarkers. A total of 35 older adults (25 females/10 males, 72±6 years) participated. The baseline assessments included: body composition, handgrip strength and a physical performance test (PPT) (gait speed, TUG, time to climb and descend 10 stairs). Leg muscle quality (LMQ) and stair climb power (SCP) were calculated. Participants performed in a randomised order a single session of 30 mins AE (cycling at 70% of peak heart rate) and RE (leg press at 70% of one repetition maximum and jumping regimen). At baseline, higher muscle strength was associated with higher P1NP and better physical performance (lower PPT score). Similarly, higher SCP was associated with higher P1NP and the beta-fragment of CTX (ß-CTX) (p<0.05). Exercise, regardless of mode, decreased ß-CTX and

tOC (all $p<0.05$), while P1NP and ucOC were not altered. Post-exercise, lower ß-CTX was associated with higher baseline muscle strength and power. Poorer baseline mobility was associated with higher ß-CTX. Independent of exercise mode, acute exercise decreased ß-CTX and tOC. Our data suggests that in older adults the relationship between muscle quality and function and BTMs is not specific to ucOC, but to BTMs in general. Furthermore, increased BTM levels were linked to better muscle function.

General conclusions: Overall, the data from this thesis strengthen the evidence for bone-muscle interaction, but mechanisms behind this crosstalk remain unclear. Larger randomised controlled trials, as well as longitudinal epidemiological studies, are required to elucidate the link between both ucOC and BTMs with muscle function, as well as with exercise-induced responses. Whether the assessment of ucOC or the ucOC/tOC ratio should be added to the standard screening in clinical care for the early identification of people who are at risk of falls and fractures needs to be evaluated further.

Student declaration

"I, Cassandra Smith, declare that the PhD thesis entitled "Uncovering the interaction between bone and muscle in older adults: effects of exercise" is no more than 80,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work."

I have conducted my research in alignment with the Australian Code for the Responsible Conduct of Research and Victoria University's Higher Degree by Research Policy and Procedures.

All research procedures reported in study 4, chapter 6 were approved by the Melbourne Health Human Research Ethics Committee, with Victoria University Human Research Ethics Committee mirror approval, HREC/17/MH/335. All other studies reported in this thesis were approved by their respective ethics committee's and have been reported in the corresponding publications.

Signature: 

Date: 30 May 2022

COVID-19 impact statement

As per most research globally, the COVID-19 pandemic took a significant toll during my Ph.D. tenure. The second half of my candidature was impacted by the pandemic, which required several modifications and changes to the current thesis. The most notable change was removal of the planned analyses from the bio-samples collected during the clinical trial relating to study 4, the Wellderly Study. Study 4 of this thesis was supposed to include an assessment of the direct effects of ucOC on human myotubes obtained from the participants. However, due to the Victorian lockdown restrictions, the biochemical laboratory access at Victoria University and access to the institution prevented any further laboratory work. All PhD students were advised by the Institute that modifications to the proposed theses were required to avoid delays to submission. The modification to this thesis plan included two additional review papers, which are presented in the literature review. There were also a range of other challenges during this period such as the transition to working remotely, from home, in an isolated environment without the ability to meet and work with the research group. In addition, it should be noted that throughout the Covid-19 pandemic impact I also became a first time mother which had significant challenges with access to usual healthcare, family and community support and created challenges for my transition back into work. Despite these challenges, I am very proud of this high quality thesis as well as my research output with multiple publications, grant successes and awards throughout this period.

Acknowledgements

The completion of this thesis would not have been possible without the support and contributions from a host of individuals. While all of these individuals have my eternal gratitude, there are some who deserve a specific acknowledgement. The individuals below have had a significant role in supporting my development throughout this journey and the completion of this thesis would not have been possible without them.

Firstly, to my supervisors Professor Itamar Levinger and Professor Gustavo Duque, who I take great pride that I have the opportunity to work with and who are leading experts in their respective research fields. **Itamar**, the past 5 years have gone by so quickly, I cannot believe how fortunate I am to have had a supervisor like you. I express my utmost and sincerest gratitude for you, a mere acknowledgement is an understatement for the amount of time, support and dedication you have given me to grow into the researcher I am today. You taught me that nothing was impossible and continued to motivate me to lift my research to the next level. You also played a significant role in nurturing me through some of my biggest transitions from a clinician come researcher and then as a researcher come mother. You gave me continual support but also gave me ample opportunity to be independent. Any success I have had in part is attributed to you, for that I am eternally grateful. **Gustavo**, thank you for the time you have given me and for you expert clinical guidance and wealth of knowledge you have contributed throughout my candidature. Your dedication to clinical practice combined with your drive and passion in research is truly inspiring, I am extremely fortunate to have worked with you.

There are a number of colleagues who also made substantial contributions to my development. A**ssociate Professor Joshua Lewis** and **Dr Marc Sim**, it has been a pleasure to learn and work with you both, your knowledge and expertise has been immensely valuable. I sincerely appreciate your dedication, time and support to me along this journey. **Professor Markus Herrmann**, thank you for the opportunity to train in your laboratory and for supporting the analyses of the bio samples collected as a part of the Wellderly study. I am very fortunate to have collaborated with you, you have contributed a wealth of knowledge and expertise to many aspects of my development as a researcher. To **Dr Alba Moreno-Asso** and **Dr Xuzhu Lin,** thank you for all of your time, training and contributions of knowledge and expertise to many aspects of this thesis. Unfortunately due to Covid-19 restrictions, the original plan for this thesis that involved

our laboratory experiments was not feasible to complete. However it would be an absolute oversight not to recognise your valuable contributions and friendship, thank you. **Dr Andrew Garnham,** none of these research projects could be possible without you, you have been such an important figure for many students. Your wealth of knowledge, positive and relaxed attitude created a calm and enjoyable research space. **Dr Mary Woessner** and **Dr Luke McIlvenna** your friendships over the many years, ongoing support and knowledge in specific research methods was invaluable in my progress as a researcher, thank you.

To **all of the research participants,** a very big thank you, none of this research could have been possible without your valuable contributions!

To **my family, and dear friends**, your ongoing support, love and care for me throughout this journey has made this PhD possible. You were there for the good times and the bad, and always encouraged and motivated me. Your patience and understanding for my commitment to this thesis has been deeply appreciated.

To **my husband Anthony**, words cannot express how grateful I am for your support and love. Anthony, you encouraged me to begin, have supported me throughout and now as I complete this journey. Your selflessness and dedication to me and this thesis have been a true pillar of support through all of the years. None of this could have been possible without you so with all of my heart I thank you.

To **my beautiful son Oscar**, who I would like to dedicate this thesis to. I love you dearly, you have taught me many things but mostly to be present and to enjoy every moment as time goes so quickly. You have grounded me and taught me a love that I never knew possible. Being your mum is the greatest gift and although you didn't know it, you sacrificed many hours of fun with me so that I could complete this thesis. Thank you my little Oscar bear.

Publications and presentations

Details of included papers: thesis with publication

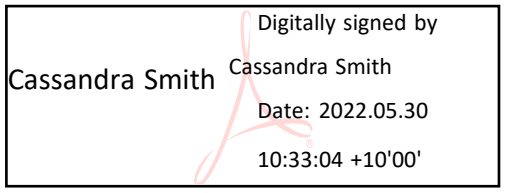
Chapter No	Publication Title	Publication Status	Publication Details
2	Sarcopenia definition: does it really matter? Implications for resistancetraining?	Published	Smith, C., Woessner, M. N., Sim, M., & Levinger, I. (2022). Sarcopenia definition: Does it really matter? Implications for resistance training. Ageing research reviews, 78, 101617. Advanceonline publication. https://doi.org/10.1016/j.arr.2022.101617 Q1 journal, SJR 1/35 Aging, IF 10.9
2	Osteocalcin- small peptide, big controversy	Manuscript ready for submission	
3	Osteocalcin and its forms across the lifespan in adult men	Published	Smith, C., Voisin, S., Al Saedi, A., Phu, S., Brennan-Speranza, T., Parker, L., Eynon, N., Hiam, D., Yan, X., Scott, D., Blekkenhorst, L.C., Lewis, J. R., Seeman, E., Byrnes, E., Flicker, L., Duque, G., Yeap, B. B., & Levinger, I. (2020). Osteocalcin and its forms across the lifespan in adult men. *Bone*, *130*, 115085. https://doi.org/10.1016/j.bone.2019.115085 Q1 journal, SJR 44/232 Endocrinology, Diabetes and Metabolism, IF 4.147

Chapter No	Publication Title	Publication Status	Publication Details
4	Higher undercarboxylated to total osteocalcin ratio is associated with reduced physical function and increased 15-year falls-related hospitalizations: The Perth longitudinal study of aging women	Published	Smith, C., Lewis, J. R., Sim, M., Lim, W. H., Lim, E. M., Blekkenhorst, L. C., Brennan-Speranza, T. C., Adams, L., Byrnes,E., Duque, G., Levinger, I., & Prince, R. L. (2021). Higher Undercarboxylated to Total Osteocalcin Ratio Is Associated With Reduced Physical Function and Increased 15-Year Falls-Related Hospitalizations: The Perth Longitudinal Study of Aging Women. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research, 36(3), 523–530. https://doi.org/10.1002/jbmr.4208 Q1 journal, SJR 23/232 Endocrinology, Diabetes and Metabolism, IF 6.741
5	The effects of acute exercise on bone turnover markers in middle-aged and older adults: A systematic review.	Published	Smith, C., Tacey, A., Mesinovic, J., Scott, D., Lin, X., Brennan-Speranza, T. C., Lewis, J. R., Duque, G., & Levinger, I. (2021). The effects of acute exercise on bone turnover markers in middle-aged and older adults: A systematic review. . Bone, 143, 115766. https://doi.org/10.1016/j.bone.2020.115766 Q1 journal, SJR 44/232 Endocrinology, Diabetes and Metabolism, IF 4.147
6	Higher bone remodelling biomarkers are related to a higher muscle function in older adults: effects of acute exercise	Under review	Higher bone remodelling biomarkers are related to a higher muscle function in older adults: effects of acute exercise. Date of submission: 29 Marc 2022

Declaration by:

Cassandra Smith

Signed:



Date:

30 May 2022

Details of publications not included in this thesis

Title	Status	Details
Progressive resistance training for concomitant increases in muscle strength and bone mineral density in older adults: a systematic review and meta-analysis.	Published	O'Bryan, Guiliano, Woessner, Vogrin, **Smith** et al. Progressive resistance training for concomitant increases in muscle strength and bone mineral density in older adults: a systematic review and meta-analysis. Sports Med (2022)
Osteoglycin across the adult lifespan	Published	Woessner, Hiam, **Smith** et al. Osteoglycin across the adult lifespan *J Clin Endocrinol Metab* (2021)
Higher levels of circulating osteoprogenitor cells are associated with higher bone mineral density and lean mass in older adults: a cross-sectional study	Published	Feehan, **Smith*** et al. Higher levels of circulating osteoprogenitor cells are associated with higher bone mineral density and lean mass in older adults: a cross-sectional study. *JBMR plus* (2021)
Elevated neuropeptide Y1 receptor signalling contributes to β-cell dysfunction and failure in type 2 diabetes	Published	Yang……, **Smith** et al. Elevated neuropeptide Y1 receptor signalling contributes to β-cell dysfunction and failure in type 2 diabetes. *Mol Metab* (2021)
Letter to the editor from Smith et al: osteosarcopenia in reproductive-aged women with polycystic ovary syndrome: a multicenter case-control study	Published	**Smith** et al. Letter to the editor from Smith et al: Osteosarcopenia in reproductive-aged women with polycystic ovary syndrome: a multicenter case-control study. *J Clin Endocrinol Metab* (2020)
Time to consider all osteocalcin forms	Published	**Smith** and Levinger. Time to consider all osteocalcin forms. *Hubble in a nutshell* (2020)
Association between circulating osteocalcin and cardiometabolic Risk factors following a 4-week leafy green vitamin K-rich diet	Published	Tacey, Sim, **Smith** et al. Association between circulating osteocalcin and cardiometabolic risk factors following a 4-week leafy green vitamin K-rich diet. *Annals Nutr Metab* (2020)

Title	Status	Details
Undercarboxylated osteocalcin is associated with vascular function in female older adults but does not influence vascular function in male rabbit carotid artery *ex vivo*	Published	Tacey, **Smith**, Woessner et al. Undercarboxylated osteocalcin is associated with vascular function in female older adults but does not influence vascular function in male rabbit carotid artery *ex vivo. PLOS One (2020)*
Physical activity, a modulator of aging through effects on telomere biology	Published	Semerano, **Smith** et al. Physical activity, a modulator of aging through effects on telomere biology. *Aging* (2020)
Aerobic fitness and telomere length in skeletal muscle and leukocytes across the lifespan	Published	Hiam, **Smith** et al. Aerobic fitness and telomere length in skeletal muscle and leukocytes across the lifespan. *Aging* (2020)
The effect of an atherogenic diet and acute hyperglycaemia on Endothelial function in rabbits is artery specific.	Published	Tacey, Quaradahki, **Smith** et al. The Effect of an atherogenic diet and acute hyperglycaemia on endothelial function in rabbits is artery specific. *Nutrients* (2020)
Undercarboxylated osteocalcin has no adverse effect on endothelial function in rabbit aorta or human vascular cells	Published	Tacey, Miller, Quaradahki, **Smith** et al. Undercarboxylated osteocalcin has no adverse effect on endothelial function in rabbit aorta or human vascular cells. *J Cell Physiol*, *236*(4), 2840–2849
Effects of dietary inorganic nitrate supplementation on exercise performance in patients with heart failure: a study protocol for a randomized, placebo-controlled, cross-over trial	Published	Woessner, Levinger , Neil , **Smith,** and Allen. Effects of dietary inorganic nitrate supplementation on exercise performance in patients with heart failure: a study protocol for a randomized, placebo-controlled, cross-over trial. *JMIR Res Protocol.* 2018.

Presentations arising from data in this thesis

(bolded- received awards or travel funding)

Year	Title	Type	Details
2021	Osteocalcin, muscle function and 15-year falls hospitalizations in older women: the Perth longitudinal study of ageing women	Oral	International Osteoporosis Foundation (IOF) conference, virtual
2021	Effects of acute exercise on bone turnover markers in middle-Aged and older adults: a systematic review	Poster	International Osteoporosis Foundation (IOF) conference, virtual
2021	Osteocalcin, muscle function and 15-year falls hospitalizations in older women: the Perth longitudinal study of ageing women	Oral	Australia and New Zealand Society for Sarcopenia and Frailty Research, EMCR World Sarcopenia Day, virtual
2021	Effects of acute exercise on bone turnover markers in middle-Aged and older adults: a systematic review	Poster and Virtual Poster presentation.	American Society for Bone and Mineral Research (ASBMR) Conference, virtual
2021	**Christopher and Margie Nordin finalist.** Osteocalcin, muscle function and 15-year falls hospitalizations in older women: the Perth longitudinal study of ageing women	Oral	Australia and New Zealand Bone and Mineral Society (ANZBMS) Conference, virtual
2021	Effects of acute exercise on bone turnover markers in middle-Aged and older adults: a systematic review	Poster & e'poster presentation	Australia and New Zealand Bone and Mineral Society (ANZBMS) Conference, virtual

Year	Title	Type	Details
2021	Higher bone remodelling is related to higher muscle function and lower insulin resistance in older adults: effects of acute exercise	Poster & e'poster presentation	Australia and New Zealand Bone and Mineral Society (ANZBMS) Conference, virtual
2020	**Osteocalcin, muscle function and 15-year falls hospitalizations in older women: the Perth longitudinal study of ageing women**	**Oral**	**American Society for Bone and Mineral Research (ASBMR) Conference, virtual**
2019	**Osteocalcin and its forms across the lifespan in adult men *Best Oral presentation**	**Oral**	**Australian Institute for Musculoskeletal Science (AIMSS) symposium, Melbourne**
2019	**Osteocalcin and its forms across the lifespan in adult men.**	**Poster**	**Australia and New Zealand Bone and Mineral Society (ANZBMS) Conference, Darwin**
2019	Osteocalcin and its forms across the adult male lifespan	Poster	American Society for Bone and Mineral Research (ASBMR) Conference, virtual
2019	**Uncovering the role of osteocalcin in bone-muscle crosstalk in older adults: an *in vitro* study.**	**Poster**	**EMBL Facing the future symposium, Germany**
2019	**Osteocalcin across the adult male lifespan**	**Oral**	**Institute for Sport Exercise and Active Living (ISEAL) HDR Conference, Melbourne**
2019	Osteocalcin across the adult male lifespan	Flash talk and poster	Victorian Muscle Network symposium, Melbourne

Awards and grants received

(bolded- related to this thesis)

Year	Title	Type	Details	Total ($)
2022	**Bone and the transcriptome: Determining the role of osteocalcin in glucose homeostasis and chronic disease prevention (chief investigator)**	**Grant**	**Deakin Institute for Physical Activity Seed Grant**	**$15 000**
2021	**Student Research Grant for HDR Students**	**Grant**	**Victoria University, Institute for Health and Sport**	**$4000**
2021	**ASCPRI Research Methods Grant for HDR Students**	**Grant**	**Victoria University, Institute for Health and Sport**	**$1000**
2020	**Exercise as a tool to identify novel biomarkers for healthy ageing. (chief investigator)**	**Grant**	**Place-Based Planetary Health Research Grant Program**	**$27,500**
2020	**Bone-muscle-fat interaction: identifying novel biomarkers for performance and health. (chief investigator)**	**Grant**	**Defence Science Institute**	**$50,000**
2020	**Young Investigator Award**	**Award**	**American Society for Bone and Mineral Research**	**$1 000**
2019	**Uncovering the therapeutic role of osteocalcin in hyperglycemic settings: effect on older adult's myotubes. (CI)**	**Grant**	**Diabetes Australia (DARP)**	**$59,747**
2019	**Travel Grant**	**Grant**	**Australian and New Zealand Bone and Mineral Society**	**$400**

Year	Title	Type	Details	Total ($)
2019	**Travel Grant**	**Grant**	**EMBL Australia**	**$2000**
2019	**Travel Grant**	**Grant**	**Australian Institute for Musculoskeletal Sciences**	**$2000**
2019	Exercise Right for Active Ageing (ERAA) Grant, (principal applicant)	Grant	Exercise and Sport Science Australia	
2019	**Best oral presentation**	**Award**	**Australian Institute for Musculoskeletal Sciences Symposium**	**$200**
2019	**1st Place, best short-talk oral presentation**	**Award**	**Victoria University, Higher Degree by Research Conference**	**$200**
2018	**Bone-muscle interactions: Novel approaches to prevent sarcopenia in older adults (chief investigator)**	**Grant**	**Australian Institute for Musculoskeletal Sciences, Seed Grant**	**$10 000**
2017	**Uncovering the interaction between circulating osteoprogenitor (COP) cells and osteocalcin in older-adults with sarcopenia: effects of exercise (chief investigator)**	**Grant**	**Tom Penrose Community Service Grant, Exercise and Sport Science Australia**	**$16 000**

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List of abbreviations

1RM	One repetition maximum
ACSM	American College of Sports Medicine
ADLs	Activities of daily living
AIMSS	Australian Institute for Musculoskeletal Science
ALP	Alkaline phosphatase
ASM	Appendicular skeletal muscle mass
AWGS	Asian Working Group for Sarcopenia
B-ALP	Bone specific-alkaline phosphatase
BMD	Bone mineral density
BMI	Body mass index
BTMs	Bone turnover markers
BMU	Basic multicellular unit
cOC	Carboxylated osteocalcin
CTX	C-terminal telopeptide of type I collagen
CVD	Cardiovascular disease
Dkk1	Dickkopf-1
DXA	Dual energy x-ray absorptiometry
ESSA	Exercise and Sports Science Australia
EWGSOP	European Working Group on Sarcopenia in Older People
FNIH	Foundation for the National Institutes of Health
GPRC6A	G protein-coupled receptor class c group 6 member A
HIIT	High intensity interval training
HR	Heart rate
ICFSR	International Conference on Frailty and Sarcopenia Research
ICTP	Cross-Linked carboxyterminal telopeptide of type I collagen
IGF-1	Insulin-like growth factor 1
IL-6	Interlukin-6
IWGS	International Working Group on Sarcopenia
KO	Knock out
LMQ	Leg muscle quality
MAFbx	Muscle atrophy F-Box
mTOR	Mammalian target of rapamyacin

MuRF-1	Muscle ring finger protein-1
NTX	N-terminal telopeptide of type I collagen
O_2	Oxygen
OC	Osteocalcin
OPG	Osteoprotegerin
PICP	Procollagen I carboxyterminal propeptide
P1NP	Procollagen of type I propeptide
P13K	Phosphatidylinositol-3-kinase
QOL	Quality of life
RANKL	Receptor activator of nuclear factor k-B ligand
RT	Resistance training
SCL	Sclerostin
SCP``	Stair climb power
SDOC	Sarcopenia Definitions and Outcomes Consortium
TRAP	Tartrate-resistant acid phosphotase
T2D	Type two diabetes
tOC	Total osteocalcin
TUG	Timed up-and-go
ucOC	Undercarboxylated osteocalcin
VO_{2Peak}	Peak aerobic capacity
VU	Victoria University
WH	Western Health
WHO	World Health Organisation
WT	Wild type

Chapter 1: Introduction

Adults reach their peak muscle and bone mass at approximately the third decade of life, after which an age-related loss of skeletal muscle and bone mass occurs (1, 2). However, under some certain conditions, which remain unclear, this loss of bone mass (leading to osteoporosis) and muscle (leading to sarcopenia) becomes accelerated. Emerging evidence suggests that this parallel loss of bone and muscle mass and strength is driven, at least in part, by bone-muscle crosstalk (3, 4). The skeleton (bone) and skeletal muscle are closely linked anatomically, chemically and metabolically, and function in an endocrine and paracrine nature (3, 5, 6). The exact mechanisms involved in bone-muscle crosstalk remain partially explored but may include bone derived hormones (6, 7).

Osteocalcin (OC), an osteoblast-specific secreted protein, is the most abundant non-collagenous protein found within the bone matrix and is used in a clinical setting as a bone turnover marker (BTM) (8-10). Within the circulation serum total osteocalcin (tOC) exists in two forms: γ-carboxylated (cOC) and undercarboxylated OC (ucOC) where ucOC lacks γ-carboxylation at one or more sites (9, 11). Both forms are understood to be involved in different underlying processes. The cOC form is predominantly located in bone involved in bone mineralisation, whereas ucOC, which is considered the bio-active form of OC, may participate in glucose metabolism and be involved in regulating muscle mass and strength (6, 12-16). Previous studies report that circulating tOC is highest in early adulthood and lower in mid-life but in older adults the data is conflicting, as some report higher whereas others report lower values (17-25). Despite differences in the biological functions of the OC forms, few studies reported the levels of both forms, with the majority of studies focused on tOC. Consequently, the ageing effect and normal ranges of OC forms and ratios (ucOC, cOC, ucOC/tOC, cOC/tOC) are not known.

The protein ucOC is involved in energy metabolism and may be important to maintain muscle mass and strength (15, 16, 26, 27). OC-deficient mice are shown to have lower muscle mass and strength (16). Additionally, lower ucOC following hind limb immobilisation in mice is associated with reduced muscle mass and muscle force (27). Treatment with ucOC is shown to increase the cross-sectional area of extensor digitorum longus (EDL) and improve grip strength in mice, and stimulate myotube formation in C2C12 myoblast cultures *in vitro* (12). In humans, even an acute single-session of exercise can increase ucOC levels (28-31), which has been shown to be related to better glucose control (29, 32). The link between ucOC with muscle mass and strength in humans is less

clear and often contradictory (26, 33-37). For instance, some report a higher ucOC/tOC ratio is correlated with higher muscle strength in older women (26). In contrast, others report no relationship between ucOC levels and muscle mass in older adults (37). However, the evidence to date in humans is based on data that were observational, with the majority cross-sectional in design. As a result, it's unclear whether ucOC is associated with muscle mass and muscle function (strength and physical performance) in humans. It's also unknown whether ucOC is related to long-term risk for hazardous outcomes such as injurious falls.

Bone and muscle are intimately linked and both organs are regulated by mechanical loads such as exercise. Evidence suggests that bone mass may be tightly linked to skeletal muscle-derived mechanical loading (38-41). As such, underlying muscle physiology (relating to the value of muscle mass and function) may be linked to the circulating levels of BTMs (40, 42). Most of the mechanistic evidence to date has focused on ucOC (16, 27, 43) but the link between BTMs and muscle includes many factors other than just ucOC (44, 45). Other BTMs used clinically to predict fracture risk, such as c-terminal telopeptide of type I collagen (CTX) and procollagen of type I propeptide (P1NP) (46), may also be involved in this specific aspect of the bone-muscle relationship. However, it is not clear if the relationship between BTMs and muscle function is just specific to ucOC or BTMs in general, as data is inconclusive (26, 47, 48). Bone turnover is a complex process, but given BTMs are already used clinically, their potential use for identifying those at risk for low muscle mass and function remains underexplored.

In support of the bone-muscle link, it is known that long-term and acute exercise can modulate BTMs (49-52). However, most acute exercise studies are performed in younger populations, with the data in older adults providing indeterminate results. Due to the known ageing effects on the musculoskeletal system, responses of BTMs to acute exercise in older adults are likely to be different compared to younger individuals and may represent a different underlying pathophysiology. Notably, there is a very limited number of studies performed in older adults and even fewer that examine the effects of resistance exercise (49, 53-56). Currently, there is no systematic review of the literature that analyses the overall responses of BTMs to acute exercise in older adults.

The primary aims of this thesis were a) to determine the ageing effect on OC forms and ratios and whether the relationship of ucOC with muscle mass, strength and physical performance is related to long term risk for injurious falls and b) to uncover whether the

relationship with muscle function is limited to ucOC or whether other commonly used BTMs such as CTX and P1NP are involved. This thesis includes four studies. **Chapter 3** (***Study 1***) determines the ageing effect on the OC forms and ratios. **Chapter 4** (***Study 2***) explores the longitudinal relationship of ucOC and ucOC/tOC with physical function and long-term risk for injurious falls-related hospitalisations. **Chapter 5** (***Study 3***) includes a systematic review of acute exercise studies to determine BTMs responses in adults over 50 years of age and whether these BTMs responses are specific to age, sex, exercise mode and intensity. **Chapter 6** (***Study 4***) includes a randomised controlled trial of adults over 60 years of age, to determine BTM responses to acute aerobic and resistance exercises. This study also investigates the cross-sectional relationship between BTMs and muscle function and whether baseline function is related to BTM responses following exercise.

Chapter 2: Literature review

This literature review comprises two main sections. **Section 2.1** discusses the age-related effects on the musculoskeletal system. **Section 2.2** describes the interaction between bone-muscle and discusses the evidence of BTMs as possible mediators of this cross talk.

This literature review comprises a published manuscript and a manuscript that is ready for submission

Section	Manuscript details
Section 2.1	Title: Sarcopenia definition: does it really matter? Implications for resistance training? *Full citation:* Smith et al. (2022). Sarcopenia definition: Does it really matter? Implications for resistance training. *Ageing research reviews*, *78*, 101616 https://doi.org/10.1016/j.arr.2022.101617).
Section 2.2.2.1	Title: Osteocalcin- small peptide, big controversy This manuscript is ready for submission.

For the purposes of this thesis, both of these manuscripts have been embedded within the literature review.

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DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

1. PUBLICATION DETAILS (to be completed by the candidate)

Title of Paper/Journal/Book: Sarcopenia definition: Does it really matter? Im[redacted] training

Surname: Smith First name: Ca[redacted]

Institute: Institute for Health and Sport Candidate's Co[redacted]

Status:

Accepted and in press: ☐ Date: [redacted]

Published: ☑ Date: 30/0[redacted]

2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be inclu[redacted]ed in the HDR Policy and related Procedures – policy.vu.edu.au.

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Signature	**Date**

3. CO-AUTHOR(S) DECLARATION

In the case of the above publication, the following authors contributed to the w[redacted]

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in t[redacted] or interpretation of at least that part of the publication in their field of e[redacted]
2. They take public responsibility for their part of the publication, excep[redacted]nor who accepts overall responsibility for the publication;

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3. There are no other authors of the publication according to these criteria;
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2.1 Ageing

Advances in technology and improvement in prevention and management of chronic diseases have resulted in humans living longer than ever before (57). In Australia, the proportion of adults over the age of 65 years rose from 5% in 1927 to 9% in 1977 and then rapidly to over 15% in 2017 (58, 59). It is projected to rise to 22% (8.8 million) by 2057 and to 25% (12.8 million) by 2097 (59, 60). Unfortunately, for many this increase in life expectancy is not always accompanied by increases in healthy life years. Commonly, it is accompanied by an increase in disability, increased risk of chronic diseases, decline in the capacity to perform activities of daily living (ADL) and poorer quality of life (QOL) (61). The age-related alterations that occur within the musculoskeletal system play a major role in the deteriorating health and wellbeing of older adults. Alterations in hormone levels, menopause, immobilisation and disease are a natural consequence of ageing but under certain conditions can become accelerated—a process which remains poorly understood. The age-related loss of muscle mass and strength when exacerbated beyond acceptable age-related norms is termed sarcopenia, and similarly the loss of bone mass and quality is termed osteoporosis (62, 63). Both are independently related to a reduced capacity to perform ADLs, poorer mobility and the ability to maintain independence in late life (64).

2.1.1 Ageing effects on the musculoskeletal system

One of the most consistent changes notable to the musculoskeletal system with advanced ageing is the decline in muscle mass and strength and bone mass and strength (***Figure 2.1***) and concomitant increase in fat mass (1, 2, 65), which, have important functional and metabolic consequences.

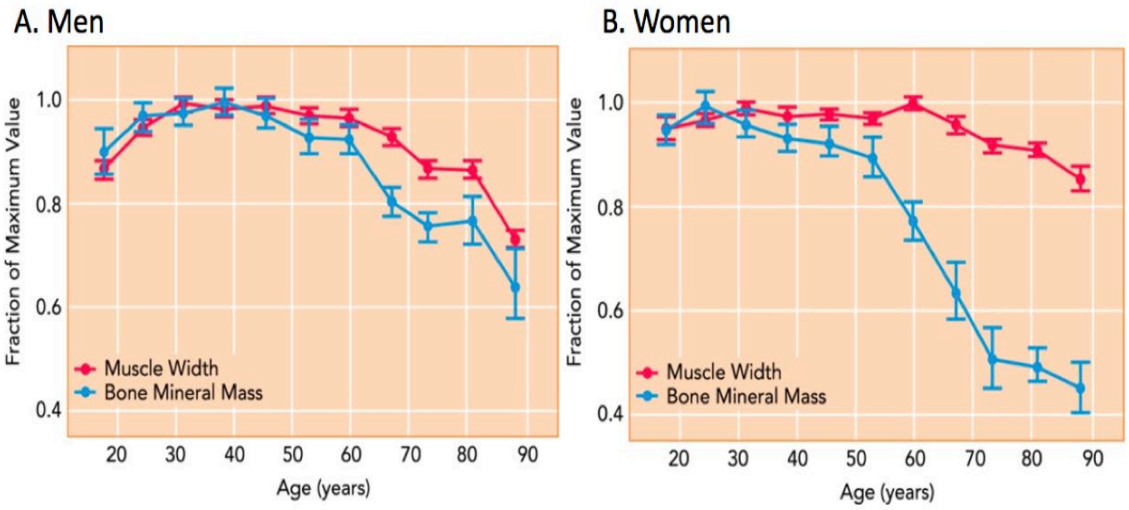

Figure 2.1: Age-related changes in bone (radius) and muscle (forearm) in adults (data normalised, demonstrating relative changes) Figure sourced from Novotny et al. (3) who had adapted the figure from Meema et al. (66).

During growth, muscle mass and bone mass increases, peaking at about the third decade of life (1, 2). Muscle mass is maintained for about two decades. Thereafter, it begins to decline from around the fifth decade of life (2). The estimated rate of loss per year of muscle mass varies between 0.4% to 2.6% (67). Some studies suggest this mostly occurs in the lower body (68). Over the lifespan, adults can expect to experience about a 30% reduction in muscle mass and 20% decline in the cross-sectional area of muscle, which is thought to be attributed to a decline in muscle fibre size and number (*described in 2.1.2*) (69, 70). Changes in age are responsible for approximately 26.4% and 15.5% of the variance of appendicular lean mass (ALM) in males and females, respectively (71). Studies suggest that muscle strength declines by 1.5% per year between 50 and 60 years of age and by 3% per year thereafter (72). Some longitudinal data demonstrate that this decline in muscle mass, strength and power begins as early as 35 years of age (69). Muscle strength and power decline to a greater extent than muscle mass, accounting for much of the disability and functional limitations associated with these alterations rather than muscle mass *per se* (***Figure 2.2)*** (69, 73-75). Some reported a loss of muscle strength of about 10-15% per decade up to age 70 years, then 25-40% per decade thereafter (75, 76). This loss in both muscle mass and muscle function when lower than the normative range expected based on an individual's age and sex or lower than an expected threshold or cut-point level, increases the risk of sarcopenia and falls and fractures (77-79).

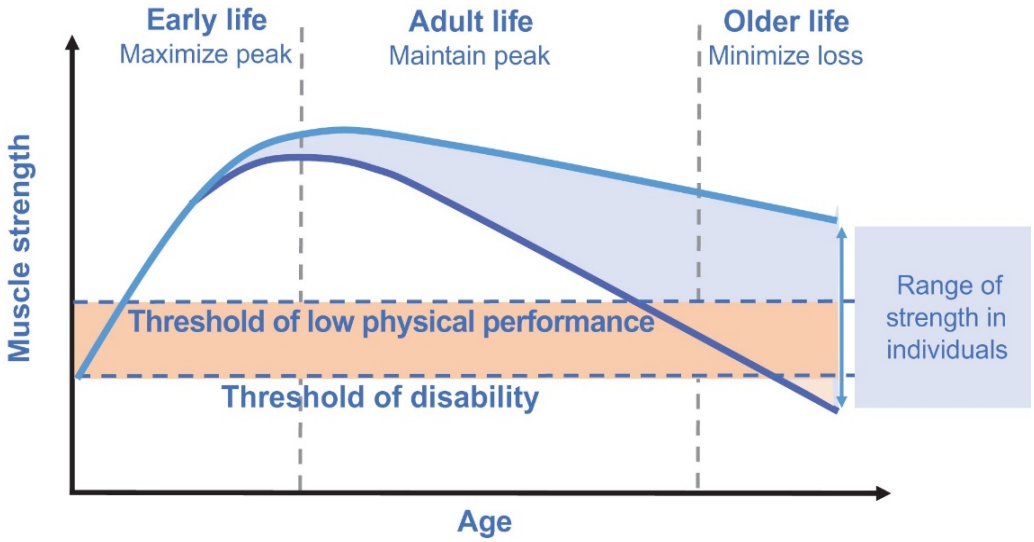

Figure 2.2 Muscle strength across the lifespan. To prevent or delay sarcopenia, the focus should be to maximise muscle in young adults, maintain muscle in middle age, and minimise loss in older adults. Figure sourced from Cruz-Jentoft et al. (77).

Bone loss occurs as a result of the normal ageing process in both sexes, once peak bone mass has been achieved (1). Once peak bone mass is obtained (***Figure 2.1***), a progressive decline in bone mass and integrity begins usually from approximately the third or fourth decade but is more severe during menopause in females (80-83). Both males and females experience a steep decline in bone mass but this occurs earlier in females at around 65 and 69 years of age as against around 74 and 79 years in males (84). The loss of trabeculae bone mass occurs earlier than the loss of cortical bone in both sexes and progressively declines with advancing age (85). This loss of both BMD and bone microarchitecture increases the risk of osteoporosis, falls and fractures in older adults, and is related to poorer QOL and early mortality (85-91). Osteoporosis affects approximately 23% of women and 6% of men greater than 50 years of age, increasing to about 43% and 13%, over 70 years of age (92, 93). While its prevalence is probably underestimated due to its asymptomatic nature, the worldwide estimates are that one in three females and one in five males greater than 50 years of age will experience an osteoporosis-related fracture (86, 94).

2.1.2 Ageing and muscle mass and function

Skeletal muscle, accounting for approximately 40 to 50% of total body mass, plays a fundamental role in human physiology enabling movement and locomotion (95). It is a major site of metabolic activity and glucose disposal and is the largest protein

reservoir in the body (a source for amino acids and glucose) (95, 96). Muscle can also secrete myokines with autocrine, endocrine and paracrine effects supporting metabolic functions of other tissues and organs (described further in **section 2.2.1**) (97, 98). Skeletal muscles exists in various sizes and shapes with different functions, but consists of about 85% muscle fibres (the remaining 15% is connective tissue) which can be broadly broken down into predominantly two main types: slow twitch (type 1) and fast twitch (type 2A and 2X) fibres (99). Slow twitch fibres are more suited to submaximal and continuous type activities as they are more efficient at using oxygen to generate energy and more resistant to fatigue. Fast twitch fibres use anaerobic metabolism to generate energy and fatigue quickly but can generate short bursts of speed and strength rapidly, suited to power activities of short duration. Age-related muscle atrophy is characterised by a reduction in muscle fibre size (diameter) due to a loss in both protein content and muscle fibres, resulting in decreased force production and fatigue resistance (100).

Muscle fibres: Older adults have lower total muscle size related to a reduction in muscle fibres by about 40% when compared to young adults, contributing to muscle atrophy and sarcopenia (70, 101). Loss in muscle fibre size during ageing is specific to fibre type, affecting mostly type II fibres (about 10 to 40% loss in the size) while type I fibres are largely unaffected (102-104). This shift in fibre type composition can begin in early adulthood (101, 102, 105) and is partly explained by a change in physical activity requirements with age (for example older adults are less likely to participate in high intensity activities that recruit type II fibres) (106). Type II muscle fibre satellite cell content and function also reduce with increased age affecting fibre growth, repair and regeneration (103, 107). At the level of both a single fibre and whole muscle, older adults also have reduced force generation capacity (108, 109). The reduction in the force producing capacity of muscle with ageing cannot be explained by a reduction in muscle mass alone (110), but is possibly related to altered neural components such as altered neural signalling, denervation to fibres and loss of motor units (111-113).

Protein turnover: During adulthood the regulation of muscle mass and fibre size reflects the fine balance between protein synthesis (hypertrophy, muscle building) and degradation (atrophy, muscle loss) (114-116). This fine balance between protein synthesis and degradation is termed protein turnover. With ageing, there is a dysregulation in this balance, with a slow shift in favour of degradation, resulting in muscle atrophy over time (117). The molecular mechanisms that underlie skeletal muscle mass maintenance with ageing and sarcopenia involve a tight interplay of a variety of signalling pathways (***Figure***

2.4) (117, 118). Under normal physiological conditions this involves the balance of a network of signals and pathways that control and coordinate protein synthesis and degradation. Muscle atrophy occurs when protein degradation exceeds that of protein synthesis via either an increase in degradation or decrease in protein synthesis (119). The major signalling pathways involved in protein degradation are the ubiquitin-proteasome, the autophagy-lysosome and caspase-3 mediated proteolytic pathways (119, 120). Pathways responsible for protein synthesis include the phosphatidylinositol-3-kinase (P13K)/AKT/mammalian target of rapamycin (mTOR) pathways (96, 116). Maintenance or preservation of muscle mass through mediators of these pathways is what ultimately regulates skeletal muscle mass (116).

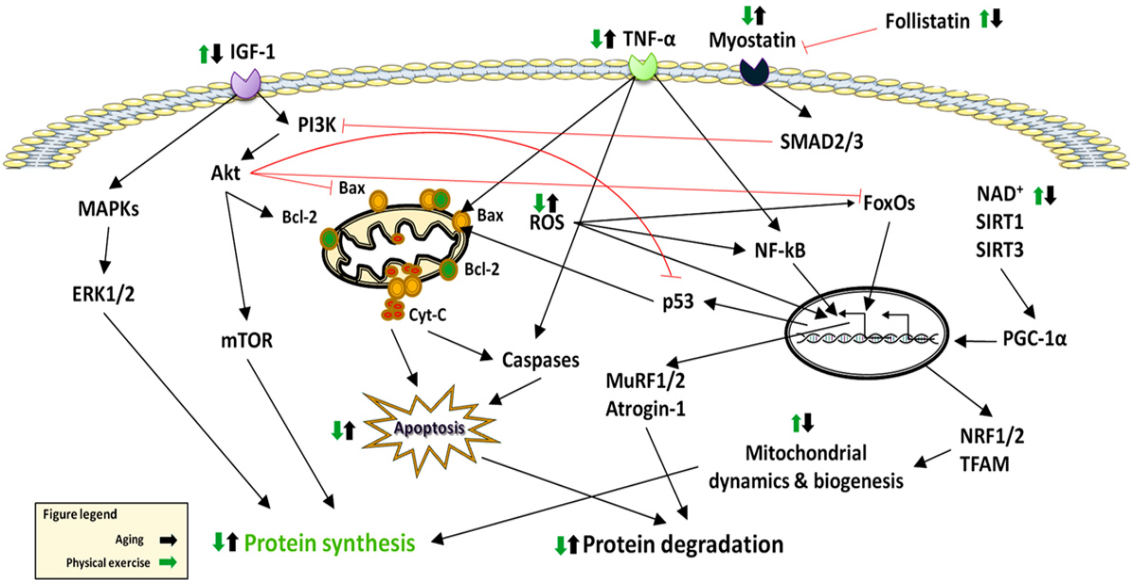

Figure 2.4 An overview of molecular pathways involved in sarcopenia. Figure sourced from Ziaaldini et al. (118).

The two major environmental influences on protein synthesis and degradation contributing to muscle atrophy and sarcopenia include the reduction in physical activity and exercise with age (decline in mechanical stress and load) and inadequate nutrition (106, 121-123) (***Figure 2.5***). In addition, ageing is also characterised by an anabolic resistance where protein synthesis responses to these anabolic stimuli (exercise and nutrition for example protein ingestion) are blunted or by an inability to suppress degradation (124-126). This may explain for example the reduced capacity to fully recover muscle loss in older individuals following periods of immobilisation such as that experienced due to injury or illness (127, 128).

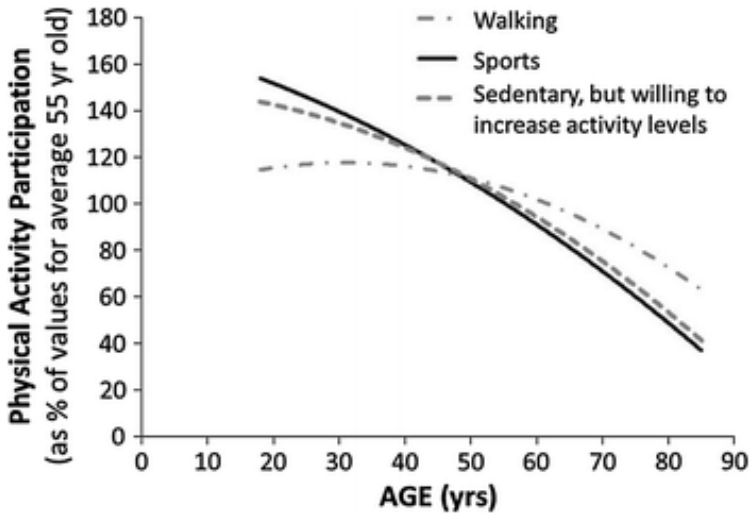

Figure 2.5 Data on physical activity participation levels in UK adults demonstrates that physical activity and exercise participation declines with increasing age. Figure sourced from McPhee et al. (106).

Moreover, evidence also supports the notion that muscle atrophy is transcriptionally regulated, where atrophied muscles are characterised by an up- or down-regulation of gene expression (120). These recognized gene expressions are termed atrogenes. The two most commonly identified atrogenes are muscle-specific ubiquitin ligases, atrogin-1/muscle atrophy F-Box (MAFbx) and muscle ring finger protein-1 (MuRF1), which are shown to be up-regulated in models of atrophy and indicate increased protein degradation via the ubiquitin proteasome system (120, 129).

Altogether, the pathogenesis of sarcopenia is complex and multifactorial, involving not only muscle atrophy but a loss of muscle function (***Figure 2.6***). In addition to normal ageing alterations, many other factors may be involved such as genetics and hereditary factors, reduced mitochondrial content and dysfunction, oxidative stress, chronic inflammation, alterations of neuronal components (loss of motor neurones), hormonal changes (e.g. insulin, testosterone, oestrogen, growth hormone, insulin-like growth factor 1 (IGF-1) and vascular dysfunction (decreased microvasculature and endothelial dysfunction) (113, 130-134). Muscle composition also changes, characterised by increases in inter- and intra-adipose tissue which is associated with reduced physical performance, mobility and balance (135-138) as well as increased muscle fibrosis, possibly related to a series of events such as injury, inflammation or tissue degeneration (139).

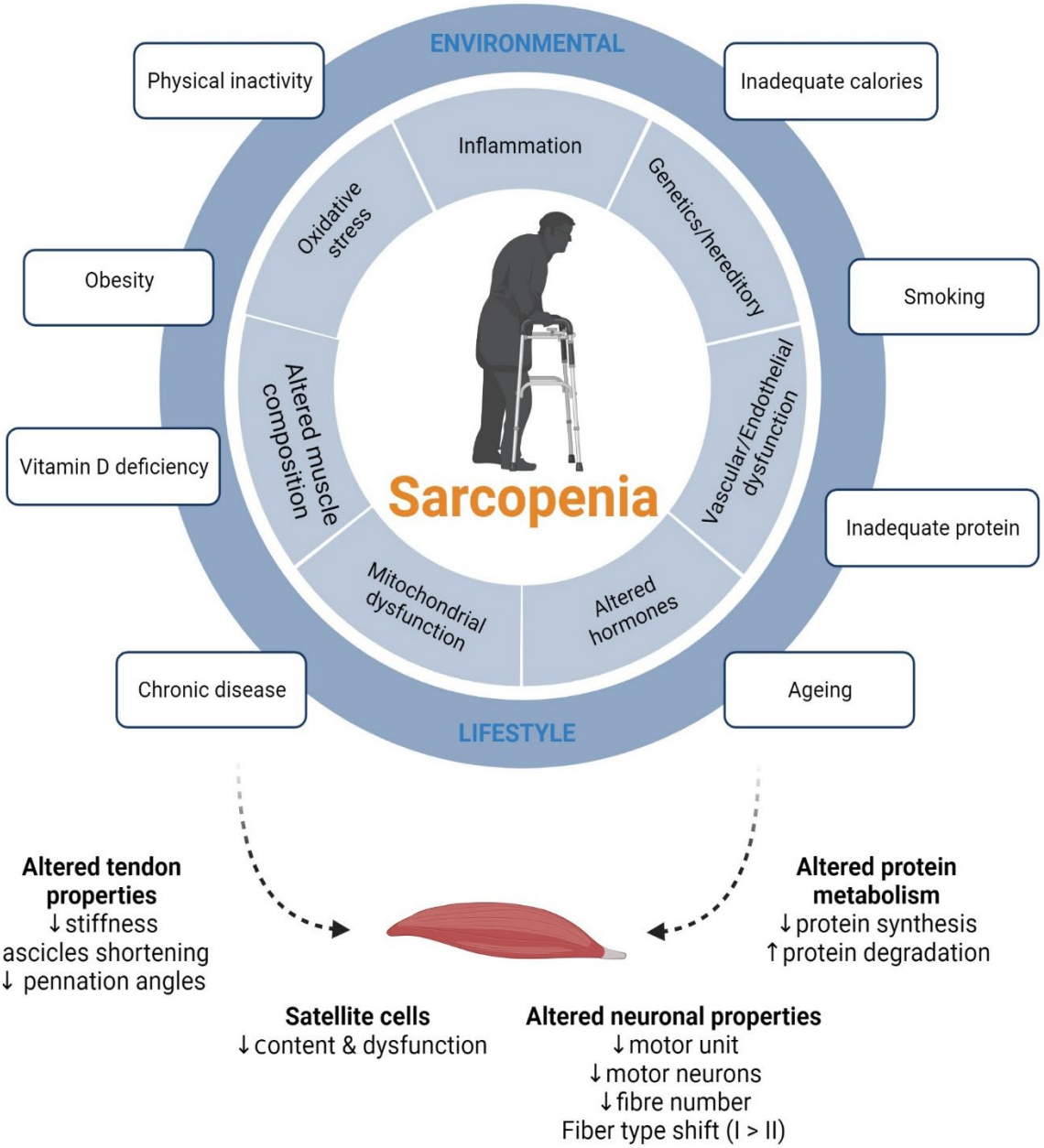

Figure 2.6 Sarcopenia pathogenesis is complex, with a number of different factors contributing to the progressive decline in muscle mass and strength with age. Created with BioRender.com

2.1.3 Sarcopenia: epidemiology

Sarcopenia and its associated health burdens have direct and indirect costs for individuals and the community (140). In the US, direct health care costs attributable to sarcopenia in 2000 were approximately $18.5 billion (141). Estimated annual costs of muscle weakness in the UK were about $2.5 billion (142). Annual health-related costs of older adults in the Netherlands in 2016, was approximately three times higher in those with sarcopenia than in those without (143). Large cohort studies in older adults have

consistently shown that the presence of sarcopenia (or its components) is related to increased risk for hospitalisation and higher health care costs compared to those without sarcopenia (140, 142, 144, 145). It has also been shown that older women with sarcopenia have a higher risk for all-cause mortality, independent of obesity (65). Given an ageing population, these figures will rise.

Above, I described possible mechanisms of muscle atrophy and other factors contributing to sarcopenia. Recently I published a review paper in *Ageing Research Reviews* which outlines and describes the numerous definitions available to define sarcopenia, including their shortcomings, and highlights the role that resistance exercise training plays in the prevention and management of sarcopenia (**Appendix 1** is the publication, Smith et al. (2022). Sarcopenia definition: Does it really matter? Implications for resistance training. *Ageing research reviews*, *78*, 101616 https://doi.org/10.1016/j.arr.2022.101617). The paper is reproduced below in *section 2.1.4*.

2.1.4 Sarcopenia definition: does it really matter? Implications for resistance training?

2.1.4.1 *Abstract*

The loss of muscle mass, strength and function, known as sarcopenia, is common in older adults, and is associated with falls, fractures, cardiometabolic diseases, and lower quality of life. Sarcopenia can also occur secondarily to chronic diseases. Recently, sarcopenia was recognized as a disease with an International Classification of Disease (ICD) code, yet, at least five definitions for its clinical identification exist. Most definitions include three themes: low muscle mass, strength and physical performance. However, the definitions vary by the number of themes needed to diagnose sarcopenia and, within each theme various parameters and cut-off levels exist. The lack of consensus on what constitutes a diagnosis can create confusion and hesitation in sarcopenia diagnosis. Currently, no pharmacological treatment exists for sarcopenia. Resistance training (RT) is safe and effective to improve muscle mass, strength and physical performance in older adults and clinical populations. Based on current guidelines, whether an individual is defined as "sarcopenic", or not, does not change the way RT is prescribed. Here, we present evidence and the inconsistencies in sarcopenia definitions and recommend that focus should be on optimizing ways to prescribe RT and increase

long-term adherence, rather than on slight modifications to sarcopenia definitions.

2.1.4.2 *Sarcopenia and its definitions: Scope of the problem*

Older adults can now expect to live to over 80 years of age (57). However, increases in life years does not always translate to healthy life years. Rather, it is commonly accompanied by disability, increased risk for chronic disease and a poor quality of life (61). The loss of muscle mass and strength and/or physical function is known as sarcopenia, depending on the clinical definition used to identify it (77, 146-149). Sarcopenia is common in older adults (> 65 years) with estimated prevalence varying between 10 to 50%, large variability in prevalence is contingent on the definition used (150, 151). Sarcopenia is commonly associated with a higher risk of falls and fractures, reduced capacity to perform activities of daily living (ADLs) and a loss of independence (78, 79). It is a multifactorial disease, and some of its risk factors include age, sex, low level of physical activity, poor diet and, chronic inflammation. As such, sarcopenia often develops in conjunction with presence of cardiometabolic disease (152, 153). Sarcopenia was recognized as a disease with its own International Classification of Disease, ICD-10 code (M62.84)(154). Although one can hypothesize that this new ICD code will promote screening for sarcopenia and therefore treatment and management, there is no consensus on its diagnostic criteria. This lack of agreement in the cut-off criteria to diagnose sarcopenia between organizations, clinicians and researchers limits the use of an ICD code, potentially complicating the effective management of the disease.

Currently, there is no universally accepted definition for sarcopenia. The validity and predictive values for adverse outcomes based on the available definitions is varied (155-157). There are at least five definitions used to diagnose sarcopenia including the European Working Group on Sarcopenia in Older People (EWGSOP2) (77); the Foundation for the National Institutes of Health (FNIH) (146); Asian Working Group for Sarcopenia (AWGS) (147); Sarcopenia Definitions and Outcomes Consortium (SDOC) (148); and the International Working Group on Sarcopenia (IWGS) (149). Of these, three represent updates to original definitions (62, 146, 158).

The criteria used to identify sarcopenia according to the five most commonly used definitions can be generally categorized into three main themes: a) muscle strength, usually hand grip strength, an assessment of upper limb strength (four of the five definitions) which has shown to have the capacity to identify older adults at risk for falls

and fractures (159-161); b) muscle mass, usually appendicular skeletal muscle mass adjusted to height or body mass index (BMI) (four of the five definitions) and c) physical performance i.e. gait speed, an assessment of mobility that has been associated with survival and predicts incident disability in older adults (four of the five definitions) (162, 163). As seen in Figure 1, even within these three themes, there are a diverse range of acceptable parameters included in each definition which may measure a different muscle characteristic. For example, muscle strength defined by EWGSOP2 includes hand grip strength, or the 5-time chair stand, an easy, portable assessment of lower limb muscle power, which has been shown to be associated with falls, frailty, slowness and functional limitation in activities of daily living in older adults (164-167). Of note, even among those definitions that include the same muscle parameter, different cut-off values are used (***Figure 2.7***). To our knowledge, there are also working groups currently formulating new definitions for sarcopenia, some of which suggest adding additional diagnostic measures including calf circumference (168), muscle density assessed by computed tomography (CT) (169), hand grip strength asymmetry (170) and perhaps even lip, tongue and suprahyoid muscle strength (171, 172). The reality of numerous definitions for sarcopenia existing (including possibly more to come) together with a lack of agreeance on cut-off levels for individual muscle parameters, leads to confusion in its diagnosis in both clinical and research settings. Inconsistent reports in the literature related to prevalence of sarcopenia are shown largely to be explained by the definitions chosen. In other words, when different definitions and cut off points are used, different results are obtained (156, 157, 173, 174). Not only does this lead to variable and inconsistent reports, it may also contribute to hesitation in a clinical setting to diagnose a patient as “sarcopenic”. The complexity in reaching an agreeable definition maybe, at least in part, be due to the fact that older adults with sarcopenia, similar to frailty, are characterized as a very heterogeneous group, which may have some practical challenges clinically (175). However, it is not just the definition that is important to identify sarcopenia, but also which health professional/s is/are responsible for its diagnosis (176). For example, dieticians, exercise physiologists and physiotherapists should all play a role. An important question that remains is whether the use of a different clinical definition for sarcopenia changes the negative outcomes associated with its progression. This should be explored in future studies.

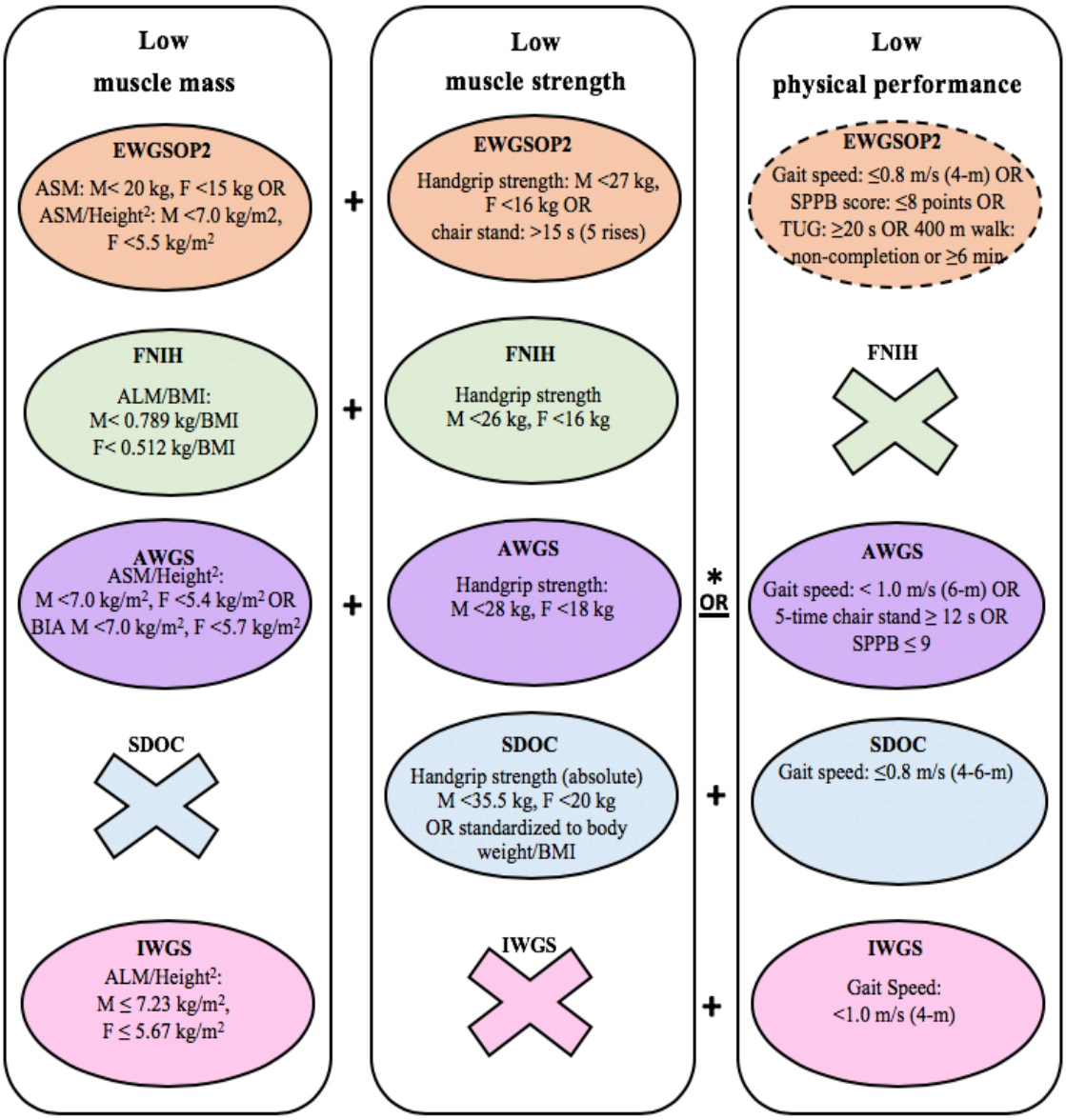

Figure 2.7 Most commonly used definitions for sarcopenia: EWGSOP2 (European Working Group on Sarcopenia in Older People); FNIH (Foundation for the National Institutes of Health); AWGS (Asian Working Group for Sarcopenia); SDOC (Sarcopenia Definitions and Outcomes Consortium), IWGS (International Working Group on Sarcopenia).

Discrepancies between agreeable cut off levels and parameters used to define sarcopenia raise an important clinical question: *is having a precise cut-off level for the diagnosis of a patient as "sarcopenic" critical for disease management?* A specific diagnostic cut-off level is undeniably pivotal for the accurate prescription of pharmacological treatment and is also valuable from a patient perspective as increased knowledge can increase self-empowerment (177). As stated by Cesari and Kuchel (175) "we must not let the perfect become the enemy of the good." In other words, additional

parameters and definitions or criteria for sarcopenia may complicate and possibly hinder the prescription of the only known effective treatment for sarcopenia, specifically, a lifestyle approach incorporating progressive resistance training (RT) in conjunction with a healthy diet comprising adequate protein and energy intake (178-180).

2.1.4.3 *Sarcopenia: what is our best defense?*

One of the most consistent changes with advanced age is the decline in skeletal muscle mass and strength. Skeletal muscle comprises ~40% of the human body weight, and its functions are widespread, including postural, mobility, energy storage and metabolism. Longitudinal data clearly demonstrates a decline in muscle mass, muscle strength and power beginning ~35 years of age (69). Notably, muscle strength and power decline to a greater extent than muscle mass, accounting for much of the disability and functional limitations associated with these age-related changes and not muscle mass, *per se* (69, 181).

Sarcopenia often presents as a comorbidity of other cardiometabolic diseases and has common risk factors (increasing age, physical inactivity, chronic inflammation and malnutrition) (152, 153, 182, 183). Many of these chronic cardiometabolic diseases (such as cardiovascular disease, type 2 diabetes, and others) also share the skeletal muscle biological characteristics of sarcopenia with alterations to muscle size (fiber number and atrophy of type II fibers, motor units), increased fat infiltration, decreased capillarization, chronic inflammation, increased oxidative stress and insulin resistance, of which, exercise has been shown to effectively mitigate (184-187). Notably, some of the loss of muscular power and function experienced by older adults and clinical populations can be attributed to age-related neuromuscular loss (113).

There are currently no approved pharmacotherapies for the treatment of sarcopenia. Phase 2 clinical trials testing the effect of an antimyostatin antibody showed minimal effect on muscle function (188). The only intervention that consistently shows to improve muscle mass, strength, and physical function in older adults is exercise training and particularly progressive resistance training (RT) (189-194). Progressive RT is a safe and effective approach to attenuate, and in some cases reverse the age-related loss of muscle mass and strength (195-197).

2.1.4.4 *Resistance exercise: the front line defense*

Progressive RT is considered a first-line strategy to prevent and manage sarcopenia (196-198). It provides numerous benefits including increasing muscle mass, strength, endurance, power and physical function, and lowering the risk of falls and associated injury such as fracture (189-192, 199-201). These are essential muscle characteristics which are required to perform ADLs in older adults and clinical populations (202-204). RT is also consistently demonstrated to be safe, effective and recommended for almost all populations including healthy older adults and those with chronic diseases such as cardiovascular disease, cancer, type 2 diabetes, osteoporosis and chronic obstructive pulmonary disease (COPD) (196, 198, 205-211). Notably, many patients with these diseases are also characterized by traits consistent with sarcopenia, with significant impairments in the capacity to perform ADLs and poorer quality of life (212, 213). According to the American College of Sports Medicine (ACSM), RT should be a component of every exercise program for healthy adults, and those with clinical conditions (211). Extensive evidence on the benefits of RT in healthy older adults and those with chronic disease is well defined, but the question remains: "*Does RT prescription change if a patient is diagnosed with sarcopenia, defined by any or different definitions*?" The answer is probably not.

2.1.4.5 *Resistance training guidelines: show me the differences!?*

The general principle of RT prescription is that the exercise programs should be progressed and individualized to each person (214). Many organizations have and continue to release independent RT guidelines for older-adults and clinical populations (198, 211). Regarding sarcopenia, while it is independently recognized as a disease, it commonly unveils as a consequence of many other chronic diseases, even despite substantial differences in pathophysiology, progression and symptoms (152, 153, 182). The pharmacological treatment of each disease vary in many ways, but the RT recommendations are similar. While it is not possible to provide an overview of all available guidelines from various organizations, herein we provide a proof of concept to demonstrate the similarities in RT guidelines across the healthy and disease continuum, using best practice clinical exercise guidelines from ACSM and recommendations for healthy individuals from both ACSM and the International Conference on Frailty and

Sarcopenia Research (ICFSR) consensus (***Table 2.1***) (198, 211). We have focused, in particular, on diseases commonly observed in conjunction with sarcopenia.

Table 2.1 Internationally recognized exercise prescription guidelines for older adults and common clinical populations that share characteristics of sarcopenia

Association (year)	**Population**	**Frequency** Days p.wk	**Exercise prescription for resistance exercise**		
			Sets	**Reps**	**Intensity**
ICFSR Consensus (2021)	Older adults	2 to 3	1 to 2	8 to 12	50 to 80% 1RM
ACSM (2017)	Older adults	2 to 3	1	10 to 15	40 to 50% 1RM
ACSM (2017)	Healthy	2 to 3	2 to 4	8 to 12	60 to 70% 1RM
ACSM (2017)	Obese	2 to 3	2 to 4	8 to 12	60 to 70% 1RM
ACSM (2017)	Type two diabetes	2	1 to 3	10 to 15	Moderate (50 to 69% 1RM) to vigorous (70 to 85% 1RM).
ACSM (2017)	Cardiovascular disease	2 to 3	1 to 3	10 to 15	40 to 60% 1RM, BORG RPE 11 to 13
ACSM (2017)	Chronic heart failure	1 to 2	2	10 to 15	40 to 70% 1RM
ACSM (2017)	Chronic kidney disease	2 to 3	1	8 to 12	65 to 75% 1RM (1RM estimated from 3RM)
ACSM (2017)	Peripheral arterial disease	2	1	8 to 12	60 to 80% 1RM
ACSM (2017)	Dyslipidaemia	2 to 3	2 to 3	8 to 12	Moderate (50% 1RM) to vigorous (75 to 80% 1RM), <50% 1RM to improve endurance
ACSM (2017)	Hypertension	2 to 3	2 to 4	8 to 12	60 to 80% 1RM *older adults 40 to 50% 1RM
ACSM (2017)	Arthritis	2 to 3	2 to 4	8 to 12	60 to 80%1RM *lower intensity for untrained (50 to 60%)

When observing ***Table 2.1***, there are noticeable similarities existing regarding resistance exercise prescription guidelines, irrespective of disease status. Regarding exercise intensity, some evidence suggests that similar increases in muscle strength have been observed when using an intensity of either moderate or heavy loads (between 40 to 90% of 1-repetition maximum, 1RM) once total volume is accounted for and if lower loads are carried out to fatigue (215, 216). In addition, comparable improvements in strength can be seen in older adults who performed two versus three days per week of RT (217, 218).

Given that a surprisingly low number of older adults currently meet exercise guidelines (219), researchers and clinicians should focus on how to engage individuals in RT that is enjoyable in order to increase adherence for long lasting benefits. The RT guidelines presented could be summarized by suggesting to complete structured exercise at least two to three days per week. Additionally, to combine whole body movements including upper and lower body exercises, of two to four sets each, and using a rep range that can be completed using a moderate to heavy intensity load until fatigue. Importantly, individualization of each component should occur regardless of disease state. This broader summary indicates that the general recommendations for RT in conditions with traits of sarcopenia do not differ substantially. The lack of variation in these guidelines also suggests that, in terms of RT guidelines, a specific disease diagnosis does not result in a major different RT recommendation.

However, it should be acknowledged that there are different approaches to RT. One of which is power training, a specific type of RT where muscle contractions are performed at high velocity. This type of exercise improves muscle power and has been associated with improved capacity to perform ADLs in older adults (220-222), in those with mobility limitations (223), and even in those that are frail (224, 225). However, power training is yet to be incorporated into RT guidelines and as such, expertise from the exercise professional and caution should be considered if it is to be used as part of a RT program. Specifically, when prescribing RT to older adults (with, or without sarcopenia traits), guidelines should be adopted as a guide to clinical practice in-conjunction with an in-depth knowledge of patient' conditions and treatments. This approach will assist with the delivery of an optimal exercise program that can be performed safely by the individual.

Indeed, the definition of sarcopenia itself may also yield a different understanding of what is being treated i.e. increasing muscle mass or/and increasing muscle strength (i.e. handgrip strength) or/and improving physical performance (reducing time to

complete the timed up and go). Furthermore, the prescription of any treatment, even exercise, requires an assessment of both risk and benefit for the individual. The risk associated with RT is typically minimal if it is prescribed within the guidelines of ACSM (211) and it is usually only associated with a delayed onset of muscle soreness (DOMs)(226). DOMs is a common experience following RT and can be experienced by individuals of all fitness levels following unaccustomed physical activity. It is typically characterized by muscle soreness and discomfort that increases with intensity within the first 24 hours following exercise, and usually subsiding within a few days (226, 227).

The benefits of RT in older adults and clinical populations with characteristics of sarcopenia go beyond the skeletal muscle level (i.e. improved strength) and includes improved capacity to perform ADLs, increased cognitive function (228, 229) and improved quality of life (230, 231). RT also reduces cardiometabolic risk factors (232, 233). For older adults who are frail, living in nursing homes or institutionalized, and often characterized by multimorbidity, the evidence for beneficial effects of RT on such outcomes is conflicting, likely due to large heterogeneity of the population as well as the definition for frailty used (234). The degree of frailty may also be critical in the effectiveness of an exercise program (234). However, benefits including improved functional outcomes (200, 229, 235, 236) and quality of life (235) have been reported in this population (237, 238). Indeed, there will be some instances whereby RT may not be suitable in particular populations due to very low function level or safety considerations. In that scenario, exercise prescription should be modified, and adapted to the physical function level of the individual taking into account comorbidities, and risk/benefit to participation.

2.1.4.6 *Other considerations*

This review focuses on RT as a treatment for sarcopenia. However, it is important to acknowledge that other lifestyle interventions may assist in muscle mass and strength preservation during ageing. This includes a balanced approach to the diet including a variety of nutritious foods from the five food groups: vegetables, fruits, grains, proteins (i.e. lean meat, fish, nuts and legumes) and dairy (milk, yoghurt etc.) (239). Prospective studies have demonstrated that when dietary protein intake is low it is linked to functional decline in older adults (240-243). Some evidence also demonstrates that adequate protein intake (>1 g/kg/day) can reduce the rate of decline in hand grip strength and mobility (244,

245). However, supplementation with protein above recommended levels in older men >65 years who were functionally limited, had no effect on muscle mass, strength or power (246). Recommendations advise that older adults (> 65 years) have higher daily protein requirements than younger adults to maintain/regain lean mass and function, and, these requirements increase for those that exercise, and are even higher again for those with acute or chronic illness (247).

Some evidence suggests a possible additive effect on muscle strength in older adults when combining protein intake with RT (178, 179, 248) or a physically active lifestyle (240). However, others do not support this link (194, 249, 250), particularly if dietary protein is already adequate prior to beginning RT (251, 252). For detailed evidence on nutritional interventions for maintaining muscle mass and strength into old age please see a recent review by Cruz-Jentoft (253).

2.1.4.7 *Where from here: Use it or lose it*

The reduction in muscle mass, strength and function is part of the ageing process, and it creates a challenge to individuals and health care systems globally. Whether an individual is diagnosed as "sarcopenic" or not has no effect on the way RT is prescribed based on current recommended guidelines. As such, the current focus on refining sarcopenia definitions, where five (or more) already exist, may in fact do more harm than good as it may add confusion in the identification of sarcopenia. Moreover, it is plausible that researchers and clinicians will handpick the definition most relevant to their needs. A large body of research demonstrates that RT and a healthy diet including adequate dietary protein and energy intake, remains the best approach in our efforts to prevent and manage loss of muscle mass, strength and physical function. It also provides broader health benefits such as risk reduction for cardiovascular and metabolic disease. From a clinical perspective, regular exercise is recognized as a cornerstone for public health, and yet, despite known health benefits, a large percentage of adults do not meet recommended guidelines (254, 255). Moreover, an abundance of studies demonstrate the importance of targeted resistance exercise, yet self-initiated participation levels are low (254, 256). Future focus for research should be aimed at understanding how to increase engagement and long-term adherence to exercise, importantly RT, to prevent functional decline and morbidity.

This previous section outlines the general exercise recommendations for older

adults and clinical populations who commonly have or are at a higher risk for sarcopenia or its components. This review informed the exercise protocol selected in the acute exercise study performed in **Study 4**. I used RE to examine bone-muscle interactions and how muscle function may influence this.

2.1.5 Ageing and the skeleton

Until recently, the skeleton was considered to have two major roles: protection and locomotion. To fulfil those two roles, the skeleton must be both strong, to protect internal organs and prevent fractures, but light, enabling locomotion (63, 257). Bone is also a mechano-sensing organ—it can sense change in individual load (body mass change), and external and environmental loads (physical inactivity), while a lack of loads (space flight, bed rest etc') increases bone loss (258). Bone also has the ability to adapt based on the loads and forces placed upon it such as that experienced during exercise (see ***section 2.4.4***) (258). Hence, it is well accepted now that bone is a self-repairing metabolically active organ, with capacity to change its mass, shape and properties in response to changes in mechanical perturbations on the system. This is achieved via a process termed bone remodelling, which refers to the cellular machinery responsible for the maintenance and integrity of bone material, composition, structure and strength. Given bone remodelling requires energy, it is suggested that to some degree bone plays a role in whole body energy metabolism (6, 259).

Bone material and composition, as well as its size and shape, is optimised according to its main function in order to act as a lever (load bearing bones) or as a spring (vertebrae shock absorbers) (63). The mineralised skeleton is defined by its outer periosteal and inner endosteal (including the endocortical, trabecular and intracortical components) surfaces. Cellular activities on these surfaces are responsible for the net balance of bone formation and resorption, and therefore the overall shape, vigour of bone during growth and ageing (63).

In young adults up to the age at which peak bone mass is achieved, bone accrual is determined by bone modelling (as opposed to bone remodelling): a well-coordinated, tightly coupled relationship between bone forming (osteoblasts) and bone resorption cells (osteoclasts) but in favour of bone formation. Once peak bone mass is obtained a progressive decline in bone mass and integrity begins as a result of changes in cell distribution with a transition into adult bone remodelling. The function of remodelling is

to repair micro-damage to bone, yet with ageing less bone is deposited than removed in each bone multicellular unit (BMU) (***Figure 2.8***) (63). In adults, this involves concentrated bone maintenance and repair, but with the balance favouring bone resorption resulting over time in bone loss, trabecular and cortical thinning, and porosity (257, 260). For reasons incompletely understood, the accelerated loss of bone mass in postmenopausal women by up to 30% (261) may be related to a decline in oestrogens (262, 263).

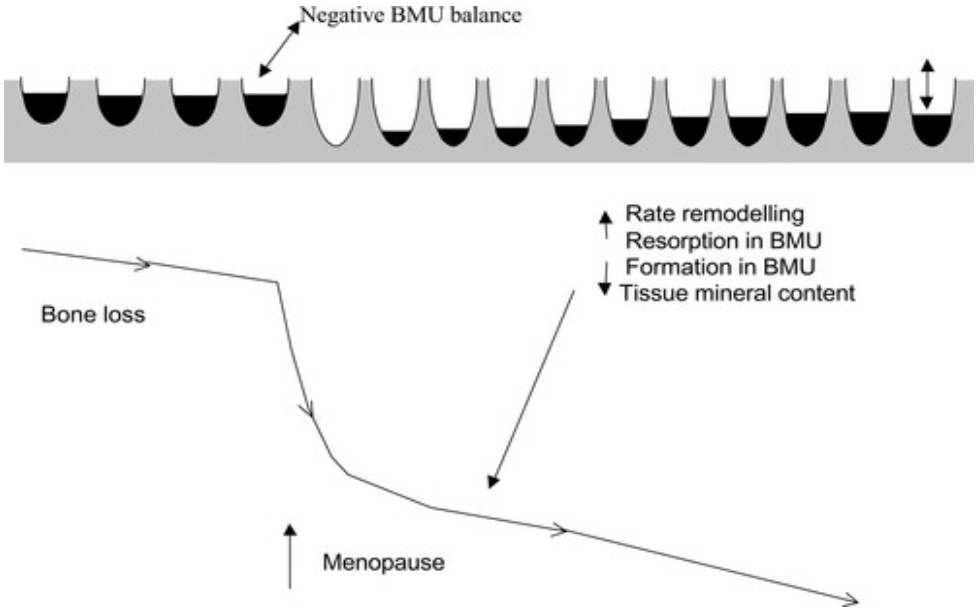

Figure 2.8 Schematic of bone remodelling leading to bone loss overtime. In young adults, material properties and structure of bone is maintained with bone remodelling (replacement of old bone with new bone). Yet with age, less new bone is formed for each site that is resorbed, producing overtime structural damage and bone loss. This process is accelerated in post-menopausal women, likely related to oestrogen deficiency. Figure sourced from Seeman (63).

2.1.5.1 *Bone metabolism*

Bone is comprised of inorganic (calcium phosphate crystals) and organic compounds (264). Its matrix comprises 90% collagen and 10% non-collagenous proteins, representing the dynamic environment where bone and external factors interact in a well-coordinated manner. Bone remodelling is a continuous process involving the coordinated actions of both osteoclasts and osteoblasts who work in team, known as basic multicellular units (BMUs), to remove and replace pockets of bone (***Figure 2.9***) (265). This process is coordinated by both osteocyte- and osteoblast-secreted factors regulating osteoclastic activity and resorption (266). Osteoclasts travel to bone surfaces via the circulation where, bone resorption begins as a result of the secretion of hydrogen ions and

hydrolytic enzymes. Osteoblastic cells communicate with osteoclasts and are recruited to resorption pits where they then secrete bone matrix proteins for the scaffolding of new bone. This interplay where osteoblasts trail behind osteoclasts within the BMU, is called coupled (267). The osteoblast secretes collagenous (predominately type 1 collagen) and non-collagenous proteins (i.e. OC) (268). The non-collagenous counterparts bind calcium and play a regulatory role in the concentration of calcium and phosphate which, are implicated in the crystallisation of hydroxyapatite on the collagen matrix. With matrix maturation, cell growth is ceased, inducing the expression of bone proteins and formation of the osteoid for mineral deposition. During this process of bone remodelling, bone releases peptides, hormones and other factors (bone biomarkers or BTMs) which are thought to reflect the underlying bone metabolism i.e. bone formation and resorption phases (269). Regulators of bone turnover includes receptor activator of nuclear factor k-B ligand (RANKL), predominately secreted by osteocytes, and osteoprotegrin, predominately produced by osteoblasts and decreases osteoclastic activity (270). Other regulatory factors also secreted by osteocytes includes sclerostin and Dickkopf-1 (Dkk1), which work to inhibit osteoblasts (***Figure 2.9***). Any alteration to these factors either increases resorption or lowers formation, contributing to osteoporosis.

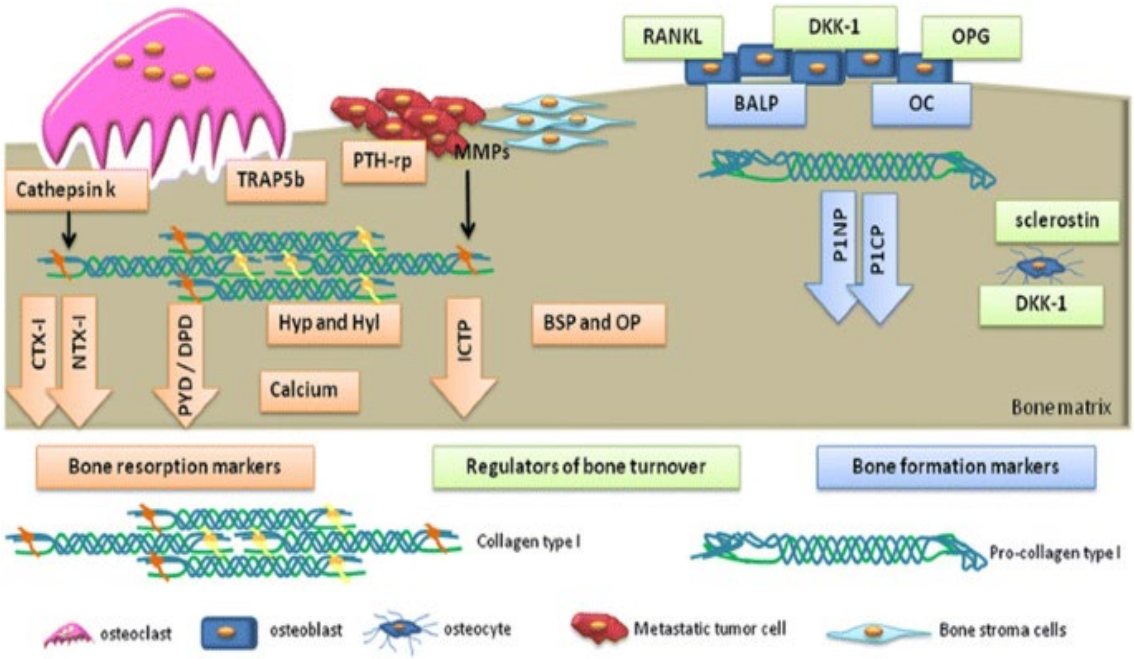

Figure 2.9 Schematic representation of bone remodelling which involves a coordinated interplay between osteoclasts and osteoblasts. The osteoclasts resorptive machinery consists of two features, a ruffled border and a resorption compartment, where the resorption compartment is formed by the attachment of the osteoclast to the bone matrix. Its ruffled border transports protons and proteolytic enzymes into the resorption compartment to acidify and dissolve minerals, degrading the bone matrix (removing bone). This process creates resorption pits that are then filled by osteoblasts. Figure sourced from Ferriera et al. (271).

The World Health Organisation (WHO) defines osteoporosis as a BMD (hip or lumbar spine) that is equal to or less than a T-score of -2.5 and, osteopenia as a T-score between -1.0 and -2.4 (91, 272). BMD is assessed via bone dual energy x-ray absorptiometry (DXA) and used to predict future fracture risk and monitor osteoporosis progression (91). BTMs are used clinically to assess fracture risk, and used in combination with DXA to improve assessment of people at high risk for osteoporosis (273). BTMs are also useful to detect rapid responses to anti-osteoporotic treatments due to their high sensitivity (46, 273). The risks associated with osteoporosis are similar to sarcopenia risk and illustrated in **Figure 2.10** (91, 274-276).

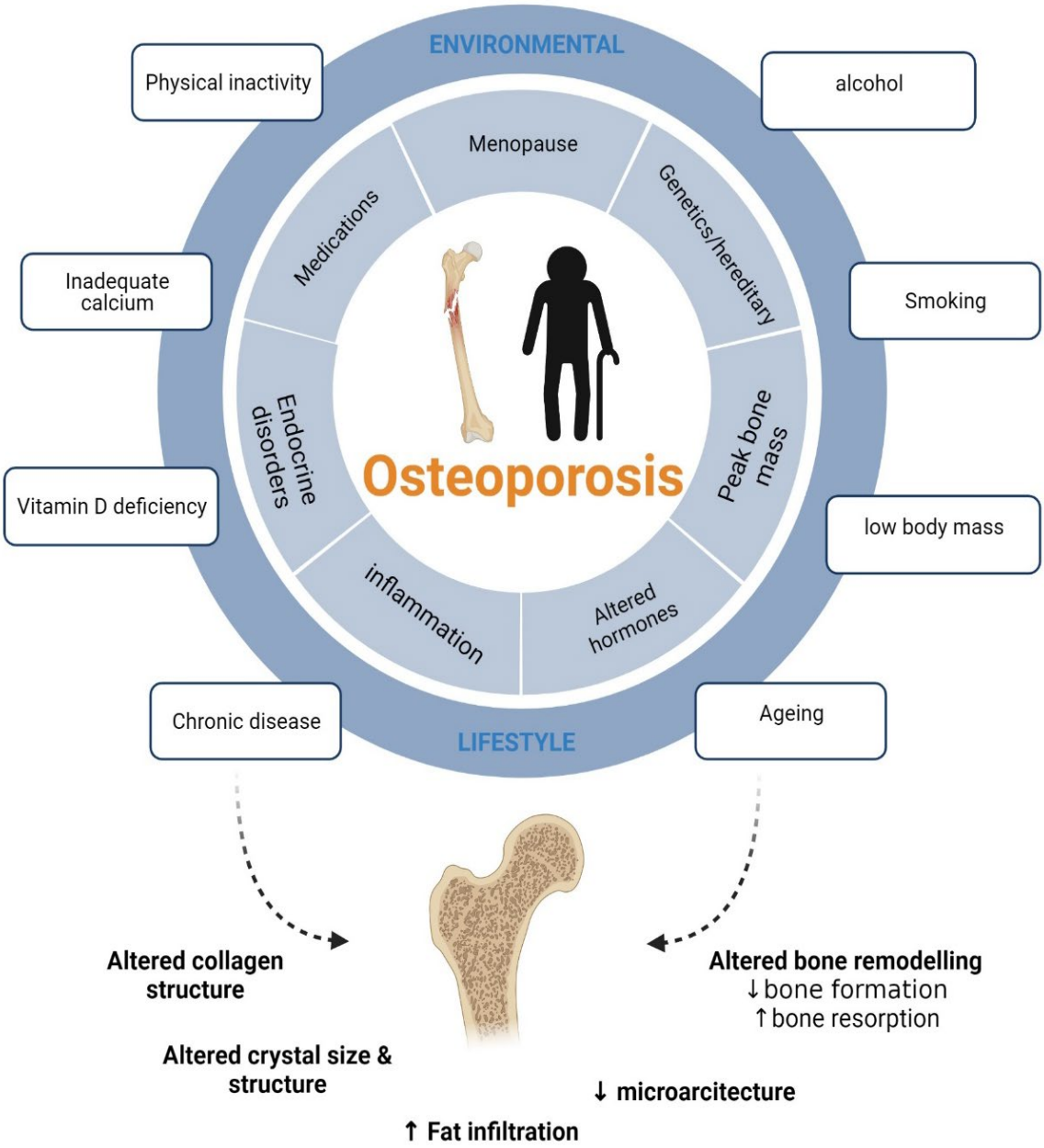

Figure 2.10 Risk factors of age-related bone loss that increase the risk for osteoporosis. Created with BioRender.com

2.1.5.2 *Bone turnover markers*

BTMs, which are measured in the circulation or urine, reflect the metabolic activity of the bone at a cellular level and can predict fracture risk independently of BMD (277). Following fracture, a heightened response of bone remodelling and increased cellular activity occurs (278, 279). BTMs are used in this instance to estimate the predicted cellular activity in favour of bone healing. Biochemical markers are broadly divided into two categories: bone resorption reflecting osteoclastic activity (degradation of products of type I collagen), and bone formation, reflecting osteoblastic activity and

by-products of collagen synthesis, matrix proteins or enzymes (273). Because the relationship between bone resorption and formation is tightly coupled, any alteration in these markers is thought to reflect a change in bone turnover. Due to their high sensitivity, BTMs are used to measure treatment responses such as osteoporotic drug treatments (280), and are commonly used in a research setting to monitor responses following for example exercise (***section 2.4.4****, and* ***Chapter 5 Study 3***). Whether BTMs are also related to muscle mass and muscle function in older adults is the focus of this thesis.

The literature contains many bone biomarkers and many of these are used clinically for the assessment of osteoporosis (46, 281). However, this thesis covers only those BTMs used throughout the exercise literature, presented in **Table 2.2**.

Table 2.2 BTMs used throughout the exercise literature

Markers of bone resorption	
C-terminal crosslinked telopeptide of type I collagen	CTX, Crosslaps
N-terminal telopeptide of type I collagen	NTX
Cross-linked carboxyterminal telopeptide of type I collagen	ICTP
Tartrate-resistant acid phosphotase	TRAP
Receptor activator of nuclear factor κB ligand	RANKL
Sclerostin	SCL
Markers of bone formation	
Alkaline phosphatase (total)	ALP
Alkaline phosphatase (bone specific)	B-ALP
Procollagen I carboxyterminal propeptide	*PICP*
Procollagen type 1 n propeptide	*P1NP*
Osteocalcin	OC
Osteoprotegerin	OPG

2.1.5.3 *Strategies to manage and treat osteoporosis*

In contrast to sarcopenia there are several effective drug treatments to maintain or improve bone mass (91). The most common are bisphosphonates (i.e. risedronate or alendronate). However, these treatments have poor patient compliance (approximately 50%) (282) related to patient perception, side effects and dosing intervals (283). Many of

these drugs also have significant side effects for example musculoskeletal pain, atrial fibrillation, osteonecrosis of the jaw, atypical fractures, gastrointestinal symptoms such as irritation to the oesophagus (284). Therefore, non-pharmacological interventions, including exercise, are an ideal strategy to maintain, preserve and improve bone health. Exercise is a cornerstone approach to prevent and manage osteoporosis, recommended by the American College of Sports Medicine (ACSM) and Exercise and Sports Science Australia (ESSA) (285, 286). A decline in caloric intake and inadequate nutrition (i.e. calcium, vitamin D and protein) are common in older adults. Consequently, adequate calcium and vitamin D are required to optimise osteoporotic treatments. In general for optimal bone health, individuals should have a well-balanced diet with adequate dietary protein, calcium, vitamin D, fruit and vegetables (287). As is the case with sarcopenia, adequate protein intake when combined with exercise training (which must include weight bearing) is the most effective approach for bone health (i.e. mass) in older adults (288, 289).

2.2 Bone-muscle interactions

No organ works independently of others. It is now clear that bone and muscle are connected not only anatomically but also metabolically and bio-chemically, both functioning in an endocrine and paracrine manner (4, 5, 7). Bone and muscle respond simultaneously to mechanical loading as well as to other stimuli (***Figure 2.11***). As previously discussed, mechanical unloading in humans such as bed rest or space flight leads to muscle atrophy, but it also has detrimental effects on bone, leading to accelerated bone loss (290-294). This simultaneous effect on both organs suggests a crosstalk exists.

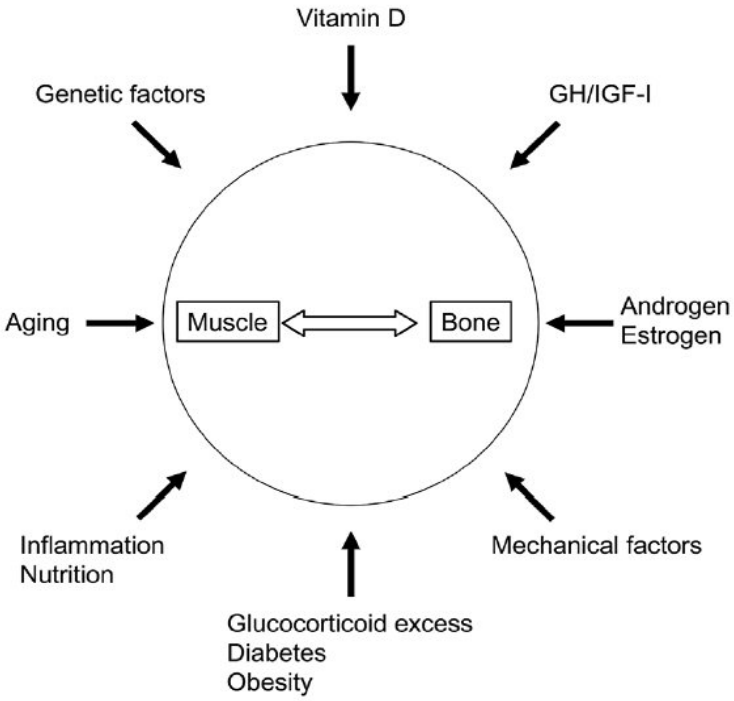

Figure. 2.11 Factors involved in bone-muscle crosstalk. Figure sourced from Kawao and Kaji (295).

In order to understand this crosstalk, researchers often target one organ and study how the other organ responds (296). In animal models this involved for example removing mechanical load (through paralysis, spinal cord injury, immobilisation, tail suspension, botox) and assessing subsequent bone changes (297-299). The working hypothesis in these models is that mechanical loading from muscle is required to load bone but changes in muscle and bone in some of these models occurred concurrently (300, 301). This suggested that the changes observed in both organs are probably activated from underlying cellular activities (a complex interaction of cellular signalling) that occur much more quickly than an observable morphological change (299). While it is easy to accept a mechanical link to explain concomitant changes in bone and muscle, the evidence suggests that changes in bone can occur independently of muscle changes (302, 303). This supports a biochemical link between the organs, where both bone and muscle are independently changed by underlying biochemical changes. Furthermore, given muscle and bone share mesodermal origin, they also share genetic determinants. Genome wide association based studies identified several genes that simultaneously affect bone and muscle with multiple overlap between traits (304). This component of the bone-muscle relationship is beyond the focus of this thesis.

To summarise, the interaction between bone and muscle is complex and probably involves many different processes. Moreover, developing evidence in the last two decades has demonstrated that bone affects muscle independently of mechanical loading via factors and hormones released by bone during bone remodelling including BTMs (5, 7, 269).

2.2.1 Bone-muscle interaction: the potential role of BTMS

Many factors are involved in bone-muscle communication (***Figure 2.12***).

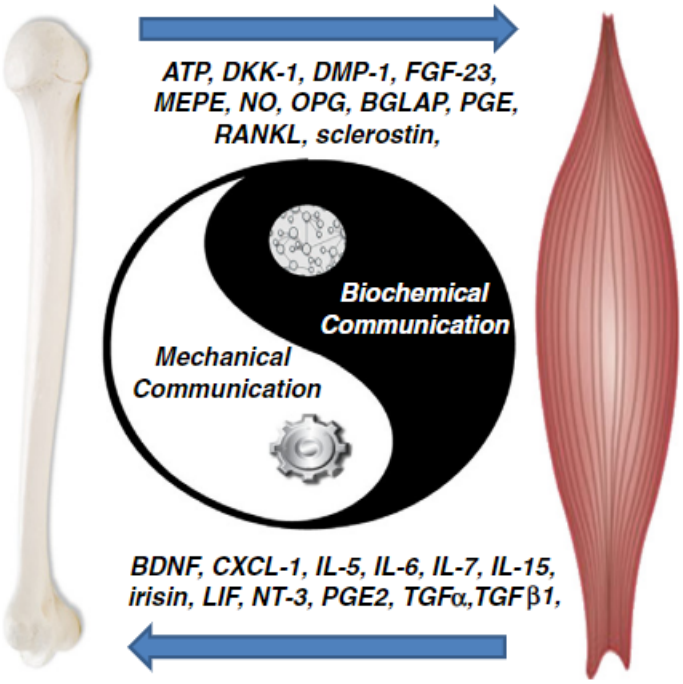

Figure 2.12 Identified muscle and bone signalling factors involved in bone-muscle crosstalk. Figure sourced from Brotto and Bonewalk (5).

Muscle – bone crosstalk: Muscle has been shown to be an endocrine organ that releases myokines affecting metabolism locally and systemically (distant organs) including at the bone and this can occur independently of mechanical loading (305). Factors such as interleukin-6, insulin-like growth factor-1, myostatin and irisin have been identified to be involved in muscle-bone crosstalk (***Figure 2.12***) (4, 5, 306). Growth factors and factors such as OGN and osteoactivin are also involved (307). In this thesis, I focus on the novel role of bone in the crosstalk with muscle.

Bone – muscle crosstalk: There is a growing list of bone-derived proteins that have been demonstrated to be involved in bone-muscle crosstalk (***Figure 2.12***) (5, 7, 308)

and includes the osteoblast secreted factor OC (6, 309). The concept of bone as an endocrine organ participating in glucose regulation was first reported in 2007 by Karsenty and colleagues (6), which led to a paradigm shift away from recognising the skeleton only for its supportive and protection roles. There are a few possible avenues by which a bone-muscle crosstalk could be facilitated, including the release of proteins and bone-derived factors into the bloodstream and the secretion of osteokines from osteoblasts and osteocytes (307). Interaction may also occur by cell-cell communication (via micro-vesicles, exosomes or extracellular vesicles) (310) or diffusion through the periosteum (small proteins <40 kilodalton, kDa) (311). As described earlier, BTMs are released by bone into the circulation during the bone remodelling process and their levels are influenced by exercise (51, 269). This thesis focuses on OC and other commonly used BTMs i.e. P1NP and β-CTX and the relationship of these BTMs with muscle mass and function in older adults, and how these markers are influenced by acute exercise.

2.2.2 Osteocalcin

The following *section 2.2.2.1* Osteocalcin – a small peptide, big controversy is a manuscript that is ready for submission. The paper is reproduced below. The purpose of this review paper is to discuss the breadth of observational and direct evidence linking OC with glucose metabolism. This earlier work was fundamental leading to the initiation of the novel investigation of the role of OC as an endocrine hormone. These investigations produced novel observations and suggested a potential role of ucOC in regulating muscle mass and function. The evidence for this novel link will be discussed in section *2.2.2.4*

2.2.2.1 *Osteocalcin – a small peptide, big controversy*

2.2.2.1.1 Abstract

Osteocalcin (OC), is the most abundant non-collagenous protein within the bone matrix. Since 2007, using mostly genetically modified animal models, it was suggested that undercarboxylated form of OC (ucOC) acts as a hormone involved in energy metabolism, male fertility, muscle mass regulation, and cognition. However, alternative OC knockout (KO) rodent models have not consistently replicated earlier findings. The aim of this review is to examine whether ucOC is linked to glucose regulation and insulin

sensitivity by examining the observational evidence in humans, as well as the evidence obtained from *in vivo*, *ex vivo* and *in vitro* ucOC treatment, independent of data collected in OC KO mice. In Overall, in humans. There is a substantial amount of evidence linking higher circulating ucOC levels with lower adiposity and decreased risk of type 2 diabetes. Furthermore, there is an increasing body of evidence that show that exogenous treatment with ucOC improves glucose regulation, both whole body and muscle glucose metabolism. Conflicting results reported may be related to methodological differences between studies, such as animals, type/source of cell-lines used, source of ucOC, treatment dose and duration. Whether the effects of ucOC on glucose regulation is clinically significant is yet to be determined.

2.2.2.1.2 Osteocalcin: overview

Osteocalcin (OC) is also known as bone gamma-carboxyglutamic acid (gla) protein. It is a small polypeptide protein of 5.7 (KDa) and the most abundant, non-collagenous, osteoblast-specific protein found within the bone matrix (9). Its synthesis occurs at the bone and it is released by osteoblasts during late stage differentiation where it is involved in bone formation and mineralization. Total serum levels of OC (tOC) are used clinically as a BTM (312). The exact role of OC in bone formation is unclear but recent data indicates it is essential for bone quality and strength (313). Following protein translation at the osteoblast endoplasmic reticulum, OC undergoes carboxylation via γ-glutamyl carboxylase at its three Glu residues (positions 17, 21 and 24) in a vitamin K-dependent manner (***Figure 2.14***) (314, 315). This post-translation modification of OC changes the conformation of this protein, thereby increasing its affinity for calcium (Ca^{2+}) ions exposed at the surface of hydroxyapatite crystal in the bone matrix (9, 316). Carboxylated (c)OC is thought to be involved in bone mineralisation (14, 313, 317). However, not all OC is fully carboxylated: a small percentage of OC remains undercarboxylated (ucOC). Following bone remodelling when bone is resorbed by osteoclasts, acidic pH causes the carboxyl groups (0 to 2) of OC to be removed (314, 315). The resulting product is referred to as ucOC, and it is considered that ucOC within the circulation is dependent on the rate of bone remodelling (314, 315). Commonly, ucOC does not readily bind to the hydroxyapatite due to its unstructured random coil, thereby inducing its leakage into the blood (318). ucOC represents up to 40% to 60% of circulating OC in humans (319). This percentage can be influenced by vitamin K intake

(10). Despite the different biological functions of cOC and ucOC to date, no study has characterised the ageing effect on OC forms and its ratios.

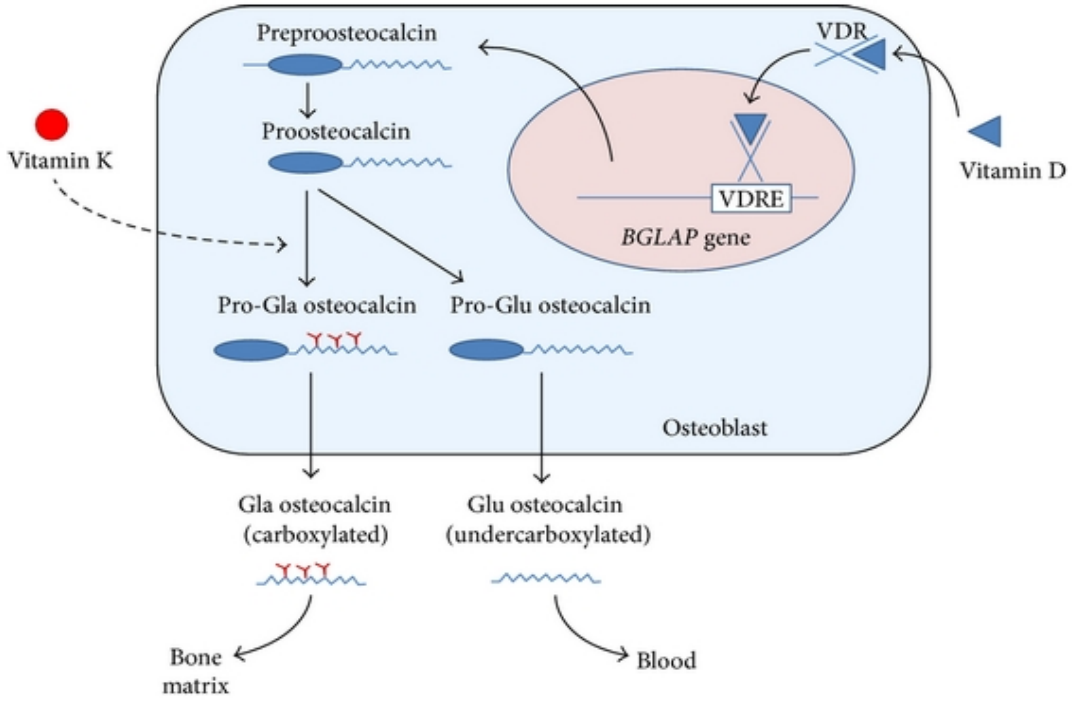

Figure 2.14 Osteocalcin synthesis in osteoblasts, a process dependent on Vitamin K. Figure sourced from Patti et al. (320).

In 2007, Karsenty and colleagues (6) were the first to report that ucOC functions as a hormone, produced in one location with action at distant locations, and therefore has the capacity to regulate energy metabolism at specific tissues and at whole-body level. They reported that OC knockout (KO) mice are characterised by reduced insulin sensitivity and glucose tolerance, while in a gain-of-function model they exhibited improved energy metabolism and resistance to diet-induced body weight gain and metabolic disorders (6). Since then, using their genetically modified mice, the Karsenty group reported that ucOC is also involved in male fertility and testosterone regulation (321), muscle mass regulation (322), brain development (323) and cognition (323) (Figure 1). These papers have generated great interest as they have opened the door for novel pharmacological approaches to treat multiple diseases such as obesity, type two diabetes (T2D) and muscle atrophy. However, their findings have not been consistently replicated in several recent studies using OC-deficient rats (324) and other models of OC KO mice (313, 317). This has led some to conclude that osteocalcin is not a hormone and

has no endocrine functions including glucose metabolism, muscle mass or testosterone production (325).

Regarding the receptor for OC, there is also some evidence that the G protein-coupled receptor class C group 6 member A (GPRC6A) is the receptor for OC (326-328) but this is controversial and not supported by others (329-332).

2.2.2.1.3 Observational evidence for OC and glucose regulation.

There is a mountain of observational cross-sectional based evidence that higher circulating levels of tOC (***supplemental Table 1, S1***) and ucOC (***Table 2.3***) are related to better glycemic control, lower BMI and fat mass in different populations (333-359). Data from a meta-analysis reported that higher tOC and ucOC are related to lower fasting blood glucose (BGL) and glycated hemoglobin (HbA1c) (360). Those with lower tOC and ucOC levels are at a higher risk for T2D compared to controls (361, 362). In fact compared to controls, tOC and ucOC levels are reported to be up to 50% lower in those who are obese, have insulin resistance or have T2D (***Table 1***) (29, 333, 334, 341, 363-375). Similarly, tOC or ucOC levels or both are lower across different clinical populations with T2D (363, 376, 377) or in conditions with abnormal glucose regulation i.e. metabolic syndrome (MetS) compared to controls (35, 336, 341, 346, 359, 364, 378-385). While the vast majority of cross-sectional evidence supports the link between lower tOC or ucOC or both with increased risk of obesity, insulin resistance and T2D, not all studies support this (347, 349-352, 386-388). For instance, in postmenopausal women no association was found at baseline between ucOC and body composition and fat mass (387). ucOC was also not associated with insulin resistance (euglycemic clamp technique) in T2D (350). Furthermore, in a different study at baseline, lower tOC and cOC, but not ucOC, were associated with higher insulin resistance (HOMA-IR)(347).

The findings are supported, however, by longitudinal prospective cohort studies and show that a relationship exists between lower tOC and ucOC with long-term risk of poorer glucose metabolism (i.e. insulin resistance, higher fasting plasma glucose) across different populations (older adults (338), older men (389), adult men (390), with a follow up range of 2 to 4 years). Moreover numerous studies show that reduced serum tOC or ucOC is related to increased risk of T2D (follow-up range of 3 to 12 years) in middle-aged adults (364, 376), older adults with high risk for CVD (348) and postmenopausal women (391, 392). However, not all studies support this association (347, 353, 388, 393-

395). For instance, recently Babey et al. (353) in a prospective eight year follow-up study in older adults (70 to 79 years) reported no correlation between baseline ucOC and incident T2D after adjustment for confounders.

Altogether the evidence is suggestive rather than conclusive and data related to ucOC are lacking with many studies only measuring tOC, probably due to methodological difficulties. Some of these observational cohort studies are also retrospective in nature or may not have been designed with tOC or ucOC as the primary outcome. This may limit the relationships.

Table 2.3 Correlative link between ucOC and glucose metabolism

First author (Ref.) Study design	**Study population** Sample size (n) Mean age (yrs)	**OC analysis method** T: time of sample C: control procedures	**OC levels** (compared to controls)	
			tOC	**ucOC**
Alfadda, (359) Cross sectional	T2D patients with and without MetS MetS n=134; Non-MetS n=69 MetS 52 yrs; Non-MetS 53 yrs	tOC N-MID Osteocalcin ELISA kit (Elecsys, Roche diagnostic Ltd., Switzerland ucOC (EIA kit, Takara) T & C: N/R	**↓ tOC MetS vs non-MetS** 8.4 (3.7) vs 9.8 (5.8) µg/L Mean (SD)	**↓ ucOC MetS vs non-MetS** 1.0 (1.0) vs 1.4 (1.7) µg/L Mean (SD)
Bullo, (396) Cross sectional and prospective	Community dwelling older men at high cardiovascular risk Taking anti-diabetics n=56 Non-antidiabetics n=23 69 yrs	tOC and ucOC (ECLIA, Roche) C; fasting	ns between groups 5.9 (3.8, 6.0) vs 6.4 (5.2, 8.0) ng/mL Geometric mean(95% CI)	ns between groups 1.7 (1.5, 1.9) vs 1.4 (0.9, 1.9) ng/mL Geometric mean(95% CI)
Chen, (397) Cross sectional	Middle-aged adults with different degrees of glucose tolerance NGT n=46; IGT n=52, 62 T2D n=62 NGT 48 yrs; IGR 50 yrs; T2D 48 yrs	tOC and ucOC (ELISA) (Invitrogen, Frederick, MD, USA, and Takara Bio Inc., Shiga, Japan, respectively), T: a.m.; C: fasting	ns between groups N/R	ns between groups N/R

First author (Ref.) Study design	**Study population** Sample size (n) Mean age (yrs)	**OC analysis method** T: time of sample C: control procedures	**OC levels** (compared to controls) **tOC**	**ucOC**
Diaz lopez (348) Prospective nested case control	Non-diabetic community dwelling adults Incident T2D=153; non-T2D n=306 66 yrs	Total OC was measured by an Enzyme Amplified Sensitivity Immunoassay Kit (DRG Instruments GmbH) ucOC (EIA kit, Takara) T: N/R; C: fasting	**↓ tOC T2D vs non-T2D** 7.5 (5.8, 9.5) vs 8.5 (6.5, 11.5) ng/mL Median (IQR)	**↓ ucOC T2D vs non-T2D** 3.6 (2.3, 5.4) vs 4.5 (2.6, 6.5) ng/mL Median (IQR)
Foresta, (368) Cross sectional	Obese and normal weight males Obese n=57; normal n=26 Obese 44 yrs; normal 39 yrs	ucOC and cOC (ELISA, Takara, Basel, Switzerland). tOC = ucOC + cOC T & C: N/R	ns between groups tOC 11.6±1.3 vs 11.9±0.9 ng/mL Mean±SEM	**↓ ucOC obese vs normal body weight** ucOC: 1.9±0.2 vs 3.7±0.5 ng/mL Mean±SEM
Funakoshi, (342) Cross sectional	Adults with varying degrees of glucose regulation Males n=34; Females n=41 Normal glucose= 25 Impaired glucose= 25 T2D = 25 65 yrs	ucOC (ECLIA, Picolumi ucOC, Eidia Co., Ltd.) T: N/R; C: fasting	Not measured	ns between groups normal: 4.3 (3.1, 5.2) ng/mL impaired: 4.0 (3.3, 6.6) ng/mL T2D: 4.0 (2.6, 5.8) ng/mL Median (25^{th}, 27^{th} percentiles)
Hwang, (398) Cross sectional	Middle aged men n=199 47 yrs	tOC and ucOC (EIA, Takara) T: a.m.; C: fasting	**Those in highest tertile of tOC had lower fasting BGL and post challenge BGL levels** 15.8(13.5, 48.0) vs 6.1(1.1, 8.1) µg/L Median(range)	**Those in highest tertile of ucOC had lower fasting BGL and post challenge BGL levels** 1.1 (0.7, 11.2) vs <0.25 µg/L Median(range)

First author (Ref.) Study design	**Study population** Sample size (n) Mean age (yrs)	**OC analysis method** T: time of sample C: control procedures	**OC levels** (compared to controls) **tOC**	**ucOC**
Iki, (399) Cross-sectional and prospective	Community-dwelling older men n=1597 73 yrs	tOC (two site IRMA, Mitsubishi, Mitsubishi Kagaku Iatron Inc.) ucOC (ECLIA, Picolumi ucOC, Sanko Junyaku Co. Ltd.) T: N/R; C: fasting	**↓ tOC in T2D vs non-T2D** 4.4 (4.2, 4.7) vs 5.0 (4.9, 5.2) ng/mL Mean (95% CI)	**↓ ucOC in T2D vs non-T2D** ucOC: 2.2 (2.0, 2.4) vs 3.0 (2.9, 3.1) ng/mL Mean (95% CI)
Iki, (373) Cross sectional and prospective	Community dwelling men T2D n=309 non-T2D n=1391 T2D= 72 yrs; non-T2D 72 yrs	tOC (IRMA, Mitsubishi, Mitsubishi Kagaku Iatron Inc.,) ucOC (ECLIA, Picolumi ucOC, Sanko Junyaku Co. Ltd.) T: N/R; C: fasting	Baseline: **↓ tOC T2D vs non-T2D** 4.3±1.5 vs 4.9±1.5 ng/mL Geometric mean ± SD	Baseline: **↓ ucOC T2D vs non-T2D** 2.1±1.9 vs 2.9±1.8 ng/mL Geometric mean ± SD
Kanazawa, (400)	Men and postmenopausal woen with T2D Males n=179, females n=149 Males 65 yrs, females 67 yrs	tOC (RIA) T: N/R; C: fasting	**↓ tOC in males vs females** 5.2±2.3 vs 7.2±3.0 ng/mL	Not measured
Kanazawa, (335) Cross-sectional	Men and postmenopausal women with T2D F n= 109; M n=180 F= 67 yrs; M= 59 yrs	tOC (RIA) ucOC (ECLIA) T: N/R; C: fasting	**↓ tOC in males vs females** 4.4±1.9 vs 7.0±3.0 ng/mL	**↓ ucOC in males vs females** 2.5±1.6 vs 4.2±3.0 ng/mL ***higher ucOC related to lower HbA1c**
Kanazawa, (401) Cross-sectional	Postmenopausal women and men with T2D not taking antidiabetics	tOC (IRMA) T: N/R; C: fasting	No difference in baseline glucose metabolism measures based on tertiles of baseline tOC	Not measured

First author (Ref.) Study design	**Study population** Sample size (n) Mean age (yrs)	**OC analysis method** T: time of sample C: control procedures	**OC levels** (compared to controls)	
			tOC	**ucOC**
Lacombe, (377)	Individuals with severe obesity Males n=11; Females n=5 42 years	tOC (ECLIA, Roche diagnostics) ucOC (ELISA, BioLegend Inc.) T: N/R; C: fasting	ns between groups N/R	**↓ ucOC T2D vs non-T2D** 2.8±0.4 vs 4.5±0.1 ng/mL
Lee, (35) Cross sectional	Post-menopausal women with and without MetS MetS m=52; non-MetS n=83 MetS: 56 yrs; non-MetS: 55 yrs	tOC N-MID Osteocalcin (ELISA, Roche Diagnostics) ucOC (ELISA, Cusabio Biotech Co., LTD) T: N/R; C: fasting	**↓ tOC MetS vs non-MetS** 15.0±6.0 vs 17.4±6.5 ng/mL	**↓ ucOC MetS vs non-MetS** 5.1±2.8 vs 6.5±3.0 ng/mL
Levinger, (29) Cross sectional	Middle aged obese men with and without T2D n=28 52 yrs	tOC IMMULITE 2000 (Siemens Healthcare Diagnostics) ucOC ECLIA (Sanko Junyaku Co., Ltd.) T: a.m. C: fasting	N/R	**↓ ucOC T2D vs non-T2D** 3.3±0.4 vs 5.7±0.7 ng/mL Mean±SEM
Liu, (379) Cross sectional	Older men with and without MetS non-MetS n=1797; MetS n=778 non-MetS 76 yrs; MetS 76 yrs	tOC (ECLIA, Roche Diagnostics) ucOC (HAP method) T: a.m.; C: fasting	ns between groups 22.1±6.4 vs 20.8±15.7 ng/mL	**↓ ucOC MetS v non-MetS** non-MetS 11.4±5.1 vs 10.9±5.9 ng/mL

First author (Ref.) Study design	**Study population** Sample size (n) Mean age (yrs)	**OC analysis method** T: time of sample C: control procedures	**OC levels** (compared to controls) **tOC**	**ucOC**
Ngarmukos, (376) nested, case-control	Adult men T2D n=63; non-T2D n=63 47 yrs	tOC (ECLIA, Roche Diagnostics) ucOC (EIA kit, Takara) T & C: N/R	**↓ tOC T2D vs non-T2D** 13.0±0.5 vs 15.2±0.5 µg/L Mean±SE	ns between groups 1.5±0.1 vs 1.1 ± 0.1 µg/L Mean±SE
Okuno, (402)	Hemodialysis patients with and without T2D T2D n=96; non-T2D n=93 T2D: 68 yrs; Non-T2D 69 yrs	ucOC (ECLIA, Picolumi ucOC, Sanko Junyaku) T: N/R; C: non-fasting	Not measured	**↓ tOC in T2D v non-T2D** 14.4 (2.7, 185.3) vs. 31.5 (2.2, 257.4) ng/mL Median (range)
Pepene, (403) Prospective case-control	Premenopausal women PCOS n=52; controls n=26 PCOS 24 yrs; controls 26 yrs	tOC (ELISA, ALPCO Diagnostics) ucOC (ELISA, Takara) T: a.m.; C: fasting, follicular phase	Ns between groups 13.76±6.58 vs 13.07±6.55 ng/mL	**↑ ucOC lean PCOS vs controls** Levels N/R
Pollock, (404) Cross sectional	Prepubertal overweight children normal glucose n=99 (51M/48F) pre-diabetes n=41 (29M/12F) normal glucose 9 yrs pre-diabetes 9 yrs	tOC and ucOC (RIA, HAP-method) T: N/R; C: fasting	**↓ tOC pre-diabetes v normal gluose** 22.8±1.5 vs 26.9±0.9 ng/mL	**↓ ucOC pre-diabetes v normal glucose** 5.6±0.7 vs 7.8±0.4 ng/mL

First author (Ref.) Study design	**Study population** Sample size (n) Mean age (yrs)	**OC analysis method** T: time of sample C: control procedures	**OC levels** (compared to controls) **tOC**	**ucOC**
Shea, (347) Cross sectional & Prospective cohort	Older adults Female n=206; Male n=142 68 yrs	tOC (RIA) ucOC (RIA, HAP method) T: a.m.; C: fasting	**Lowest tertile of tOC at baseline had higher baseline HOMA-IR** T1: 5.5±1.0 ng/mL T2: 8.1±0.7 ng/mL T3: 11.8±2.5 ng/mL Mean±SD	No difference in HOMA-IR at baseline across ucOC tertiles T1 1.6±0.7 ng/mL T2 3.3±0.5 ng/mL T3 6.1±2.3 ng/mL Mean±SD
Riquelme-Gallego, (341) Cross sectional	Patients with MetS Males n=111; Females n=124 Mean age: 64 64rs	ucOC (ELISA, Takara Bio, Japan) T: N/R; C: fasting	Not measured	**↓ ucOC MetS+T2DM vs MetS non-T2DM** N/R
Saucedo, (351) Prospective	Women with and without GDM during pregnancy and postpartum GDM n=60; non-GDM n=60 GDM 30 yrs; non-GDM 28 yrs	tOC (IRMA, Cusbio Bioassays, Codolet). ucOC (ELISA, Cusabio Biotech Co., LTD). T: N/R; C: fasting	Pregnancy- ns between groups 14.7 (9.8, 24.7) vs 17.6 (10.2, 23.5) ng/mL Postpartum; **↓ tOC GDM vs non-GDM** 24.4 (20.2, 32.6) vs 31.1 (23.8, 40.9) ng/mL	Pregnancy- ns between groups 2.9 (0.6, 5.2) vs 1.9 (0.5, 4.9) ng/mL Postpartum- ns between groups 3.0 (1.1, 8.5) vs 3.4 (1.3, 7.1) ng/mL

First author (Ref.) Study design	**Study population** Sample size (n) Mean age (yrs)	**OC analysis method** T: time of sample C: control procedures	**OC levels** (compared to controls) **tOC**	**ucOC**
Schwetz, (405) Cross sectional	Premenopausal women n=105 IR n=18 Non-IR= 87 IR 26 yrs; non-IR 28 yrs *median	tOC (ECLIA, Cobas, Roche) ucOC (HAP-method) T: a.m.; C: fasting	Baseline: **↓ tOC IR vs non-IR** 14.3 (11.6, 15.3) vs 18.0 (14.5, 24.7) ng/mL Median (IQR)	Baseline: **↓ ucOC IR vs non-IR** 2.4 (1.8, 3.5) vs 3.2 (2.1, 4.5) ng/mL Median (IQR)
Srichomkwun, (406) Cross sectional	Pregnant women with and without GDM GDM n=74 non-GDM n=56 GDM 34 yrs non-GDM 32 yrs	tOC (ECLIA, Roche Diagnostics) ucOC (ELISA, Takara Shuzo) T: N/R; C: fasting	ns between groups Non-GDM: 10.4 (7.7, 15.4) vs GDM 11.3 (8.6, 18.5) ng/mL Median (IQR)	ns between groups Non-GDM 3.9 (1.8, 8.7) vs GDM 6.1 (3.0, 9.6) ng/mL Median (IQR)
Thrailkill, (407) Cross sectional	Type 1 diabetics and age matched healthy controls TID n=115; controls n=55 T1D 18 yrs, Controls 22 yrs	ucOC (EIA kit, Takara) GLA-OC (EIA kit, Takara) T: a.m.; C: fasting	ns between groups T1DM 6.8 (0.3, 50.2) CON 5.9 (0.3, 21.1) Mean (range)	ns between groups T1DM 7.3 (0.4, 59.7) CON 6.4 (1.6, 45.6) Mean (range)
Vilafan-Bernal, (408) Cross sectional	Adults with and without T2D T2D n=80; non-T2D n=160 T2D 52 yrs; non-T2D 50 yrs	ucOC (EIA kit, Takara) Inc, Otsu, Japan T & C: N/R	Not measured	**↓ ucOC T2D vs non-T2D** ucOC: 0.2 nmol/L vs 0.3 nmol/L Median

First author (Ref.) Study design	**Study population** Sample size (n) Mean age (yrs)	**OC analysis method** T: time of sample C: control procedures	**OC levels** (compared to controls)	
			tOC	**ucOC**
Wang, (409) Cross sectional	Patients with T2D with varying degree of HbA1c High HbA1c n=36 Moderate HbA1c n=16 Low HbA1c n=11 52yrs	tOC (RIA, Beijing Atom HighTech co., Ltd) ucOC (ELISA, R&D company) T: N/R, C: fasting	**↓ tOC in those with high HbA1c vs low HbA1c** 3.9±0.8 vs 4.5±0.8 ng/mL	ucOC ns in those with high HbA1c vs low HbA1c 19.8±9.8 vs20.2±11.7 pg/mL ***those with lower ucOC have higher fasting BGL**
Yeap, (374) Cross sectional	Community-based older men T2D n=2521, non-T2D n=445 Age: 70 to 89 years (mean age N/R)	tOC (ECLIA, Roche Diagnostics) ucOC (HAP method) T: a.m; C: fasting	**↓ tOC T2D vs non-T2D** 18.6±19.5 vs 21.2±10.9 ng/mL	**↓ ucOC T2D vs non-T2D** 9.6±6.3 vs 11.2±4.7 ng/mL

Key: ↑, significantly increased; ↓, significantly decreased; ns, not significant; N/R, not reported tOC, total osteocalcin; ucOC, undercarboxylated osteocalcin; F, female; M, male; MetS, metabolic syndrome; T2D, type two diabetes; NGT, normal glucose tolerance; IGT, impaired glucose tolerance; GDM, gestational diabetes mellitus; HbA1c, glycated haemoglobin A1c; ECLIA, electrochemiluminescence immunoassay analyser; ELISA, enzyme linked immunosorbent assay; CLIA, chemiluminescence immunoassay; RIA, radioimmunoassay; EIA, enzyme immunoassay; IRMA, immunoradiometric assay; HAP hydroxyapatite

2.2.2.1.4 Interventions that manipulate ucOC levels and subsequent metabolic changes

Although there are challenges to studying direct effects of ucOC on human metabolism *in vivo*, other strategies using therapeutic and non-therapeutic approaches can be employed. These approaches alter metabolism and, albeit indirectly, ucOC (**Figure 2.15**). One such approach is to manipulate glycaemic control (i.e. hypoglycaemic drugs or lifestyle interventions e.g. exercise and diet) and observe changes in tOC or ucOC levels or conversely manipulate ucOC levels directly or indirectly (e.g. vitamin K supplementation, glucocorticoids) and observe effects on glycaemic control.

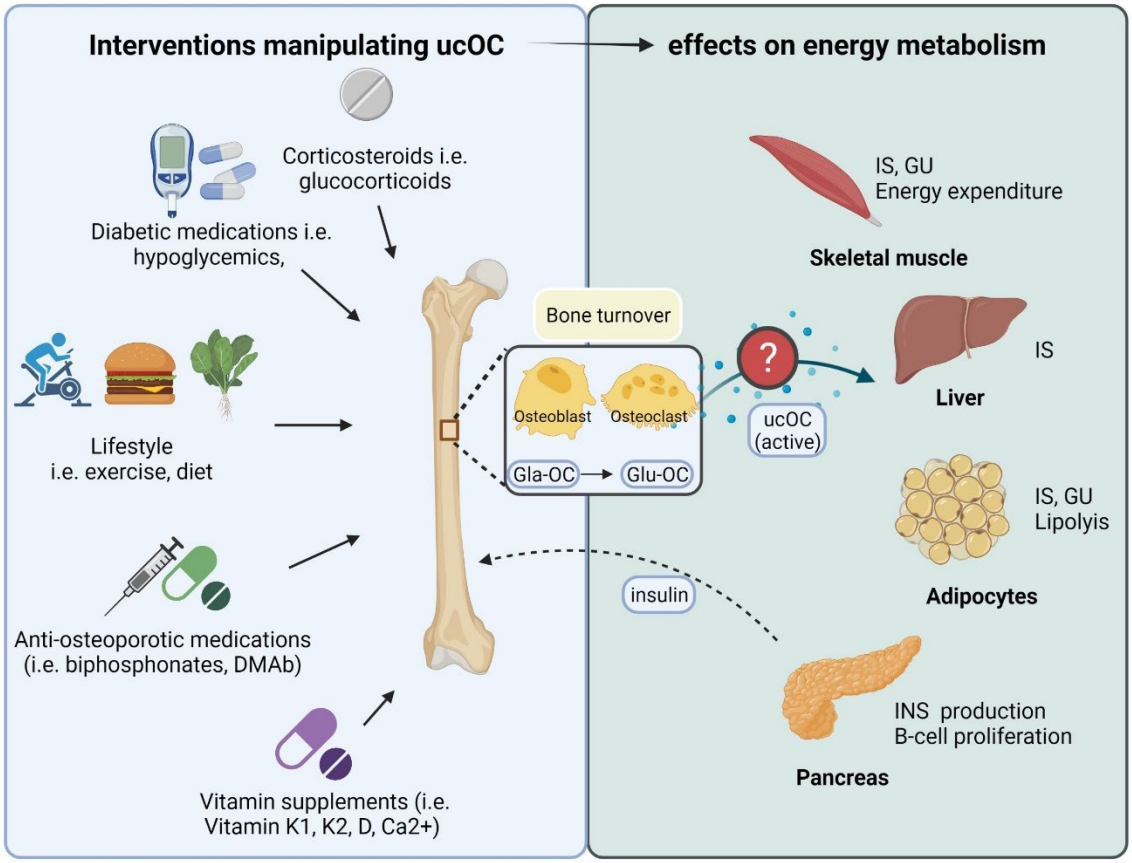

Figure 2.15 Interventions that manipulate ucOC and glucose metabolism. Created with BioRender.com

2.2.2.1.4.1 <u>Short interventions to manipulate ucOC or glucose</u>

Following an OGTT and glucagon loading test or meal, studies reported that higher tOC and ucOC levels are related to higher β-cell function and insulin sensitivity

(398, 410, 411). In middle-aged adults following an OGTT, a higher ucOC was associated with improved glucose tolerance and enhanced β-cell function (HOMA-β%) (398). The increase in glucose tolerance, insulin sensitivity and secretion was independent of adiponectin levels (410). In T2D individuals who underwent a glucagon loading test or ingestion of a meal, a higher ucOC was related to a higher C-peptide response (411).

2.2.2.1.4.2 Diabetic medications

Common methods to improve glycaemic control in patients with T2D are hypoglycaemic drugs and lifestyle interventions (diet and exercise). In those with poorly controlled T2D (HbA1c greater than 10%), hypoglycaemic medications (i.e. sulfonylurea agents, metformin, α-glucosidase inhibitor, and insulin) significantly reduced HbA1c levels which in turn resulted in increased tOC (412, 413) and decreased ucOC/tOC ratio (412). Similarly, treatment with hypoglycaemic medications in adults with T2D resulted in higher tOC and reduced glucose variability (414). Therefore, the overall observation suggests that changes in tOC are linked to changes in glycaemic control or conversely improved glycaemic control is related to higher tOC levels.

2.2.2.1.4.3 Lifestyle interventions:

2.2.2.1.4.3.1 Dietary-induced weight loss with or without exercise

Exercise and diet improve body composition (by increasing lean mass and decreasing fat mass) and increases ucOC levels which are correlated to improved body composition and glucose control (32, 415). Obese non-diabetic males with reduced body weight after following a four-month dietary program had an increased level of ucOC and a lower level of triglycerides but there were no changes in BMI, fasting blood glucose or HOMA-IR (416). Frail obese older adults after a 12 month diet (resulting in a 10% weight loss) but not exercise (multicomponent) or combination diet-plus-exercise had increased ucOC levels (around 36%) (417). Both diet and diet-plus-exercise groups improved insulin secretion but ucOC levels predicted insulin secretion in the diet group only. In a different study, sixteen weeks of dietary-induced weight loss (-7.3%) in sedentary obese women (40 to 60 years) did not alter tOC (339). However, for those in a combined diet-plus-exercise (resistance exercise) training group, post intervention tOC levels increased in parallel with an increase in leg strength and force and a reduction in fat mass. For both

groups the post intervention increase in tOC was related to decreased insulin resistance. Although slight weight loss (7.3%) in the diet group did not alter tOC, those with higher weight loss (16.8%) had increased post-intervention tOC, but this was not related to increased insulin sensitivity (339). Conversely in older women, 20 weeks of caloric restriction with and without AE (moderate or vigorous) did not alter tOC, ucOC or the ucOC/tOC ratio despite a loss in body weight and body fat (418). The women received supplemental vitamin K, vitamin D3 and calcium. Given weight loss occurred despite no change in ucOC, the researchers hypothesised that because carboxylation of OC was maintained (with vitamin K supplementation), OC is unrelated to weight loss. Other studies using Vitamin K similarly do not support the association between the change in ucOC with a change in body weight or the change in ucOC with a change in body fat (387, 419). Yet it is possible that vitamin K may assist in glucose regulation independently of ucOC, which may affect the results (420). Altogether, these studies support the notion that weight loss increases ucOC, but the data are inconsistent as to whether this is related to the favourable effects on glycaemic control induced by these interventions.

2.2.2.1.4.3.2 Exercise interventions: acute and chronic exercise

Exercise is a cornerstone approach in the prevention and management of T2D and osteoporosis (285, 286). Exercise affects bone health, in part by modulating BTMs such as tOC and ucOC (29, 30, 322, 421-423), and improves insulin sensitivity and glycaemic control. Even a single bout of exercise increases insulin sensitivity for up to 48 hours after exercise is complete (424, 425).

Acute (single-bout) AE increases ucOC in young healthy adults, middle-aged obese men and postmenopausal women (28-31). In middle-aged obese men with and without T2D, acute moderate intensity AE but not power RE (leg press plus jumping sequence) increased tOC and ucOC (29). In those with T2D, the post-exercise increase in ucOC (AE and RE groups were combined as a pooled analysis) was related to decreased post-exercise glucose levels (29). In middle-aged obese non-diabetic men, acute high intensity cycling AE increased ucOC but not tOC (30). In that study, a higher ucOC at baseline was related to higher whole body insulin sensitivity at rest and after exercise. These findings led the researchers to hypothesise the existence of a feed-forward loop whereby acute exercise increases ucOC with accompanied improvements in glucose

homeostasis and insulin sensitivity (29, 30). Exploring this hypothesis further and noting that nutrient intake and feeding (i.e. oral glucose tolerance test) lowers tOC and ucOC, the researchers undertook studies that examined whether acute exercise could attenuate the postprandial suppression of tOC and ucOC. The results showed that moderate intensity cycling and high intensity interval exercise did not alter postprandial suppression of tOC and ucOC (31, 426). However, the acute exercise bout was performed prior to insulin and glucose infusion or the OGTT and not after. In another study in middle-aged adults (427), the acute high intensity interval exercise and moderate intensity exercise was performed in the postprandial period (1h after meal consumption). However, only moderate exercise attenuated the postprandial-induced suppression of tOC and ucOC. The researchers hypothesised that the known elevation of insulin and glucose elicited by high intensity exercise may partially explain the lack of change in serum tOC and ucOC (427). Altogether the data suggest that a relationship exists between ucOC and glycaemic control and that exercise represents a tool to examine the relationship between bone, muscle and glucose metabolism. At this stage it is not known whether ucOC directly affects glycaemic control or vice versa or whether there is a direct cause and effect relationship.

Results of studies of the effects of chronic, long-term exercise on tOC or ucOC levels are conflicting. Some exercise training studies report no post-intervention change in ucOC (417), tOC (428) or both ucOC and tOC levels (28). One study reported a post-intervention increase in both ucOC and tOC levels (415) with another study reporting ucOC increases (429), tOC was not measured. Additionally, one study reported that tOC increases (430) with another study reporting tOC decreases (431) following exercise training, ucOC was not measured in these studies. However, a recent meta-analysis reported an overall increase in ucOC and decrease in glucose, insulin and HOMA-IR following training (a pooled analysis of all training modes) (32). For instance, ucOC increased in obese men with MetS following 12 weeks of high intensity interval AE, RE, or concurrent AE interval and RE (429). The increase in ucOC was associated with lower glucose, insulin and HOMA-IR. Compared to RE, ucOC was about four times higher after AE interval and concurrent sessions (5% vs approximately 25%), suggesting exercise type may have differing effects. Eight weeks of moderate intensity treadmill running in young obese males increased tOC and ucOC. This was accompanied by improved body composition as well as lower HOMA-IR and fasting plasma insulin (415). However, postmenopausal women who performed eight weeks of moderate intensity cycling had

reduced tOC and HOMA-IR with no change in insulin or glucose (431). No relationship was found between the change in tOC and change in levels of glycaemic control. This study sample was relatively small, which may have limited the correlations, and ucOC was not measured.

Altogether, it appears that dietary interventions resulting in weight loss increase ucOC levels and the increases are related to improved glycaemic control. There is some evidence that weight loss can occur, despite OC carboxylation being maintained with Vitamin K. Most data report that acute and chronic exercise increases ucOC, and this change is related to improved metabolic factors.

2.2.2.1.4.4 Vitamin K

As carboxylation of OC occurs via vitamin K it is suggested that the ucOC/tOC ratio represents a marker of vitamin K status (432-434). Therefore, one non-invasive intervention to manipulate ucOC is via vitamin K (435, 436). In healthy adults a diet rich in vitamin K is related to lower ucOC, and a lower ucOC is associated with higher HbA1c (437). Studies manipulating vitamin K (supplementation or dietary), even with very short intervention periods (2 to 4 weeks), report decreases in ucOC (436, 438-441). Some of these studies report in parallel beneficial effects on glucose metabolism using Vitamin K1 supplementation in premenopausal pre-diabetic women (440) and vitamin K2 supplementation in healthy young males (441). But other studies using Vitamin K1 supplementation (438) or consumption of green leafy vegetables (442) report no change in glucose regulation, insulin resistance or T2D development and progression despite reduction in ucOC. Conversely three months of vitamin K2 supplementation in those with T2D increased cOC and tOC without changing ucOC levels, and decreased blood glucose (443). This suggests cOC may be involved in glucose metabolism, similar to findings by others (347, 405). In these studies serum vitamin levels or dietary intakes were not always measured and therefore individual responses may be different based on baseline levels. Additionally, a dietary analysis to account for other confounding factors independent of the intervention (i.e. dietary sources of the vitamin) was not performed in many studies. Besides OC, other proteins contain glutamic acid residues and are dependent on vitamin K for carboxylation. Hence, it is possible Vitamin K supplementation has a wider effect, or perhaps a direct effect on glucose regulation via other mechanisms (420).

2.2.2.1.4.5 Glucocorticoids

Glucocorticoids (GC) are a principal treatment of chronic inflammatory disorders such as rheumatic diseases. It has been shown that GC treatment is associated with increased risk for hyperglycaemia and worsening of pre-existing diabetes or GC-induced diabetes. Additionally, those receiving GC-treatment have been shown to have lower serum levels of tOC, even at low doses (444, 445). This observed decrease in tOC levels in GC treated patients is associated with increased likelihood of presence of T2D (446). A recent study showed that GC decreased total OC and PINP in a dose-dependent manner and that these changes were related to the GC-induced adverse effects on glucose and lipid metabolism (447). It has also been shown that endogenous glucocorticoids have negative effects on muscle mass (448).

2.2.2.1.4.6 Osteoporotic treatments and glucose metabolism

Another intervention that supresses bone remodelling is antiresorptive therapy (i.e. bisphosphonates), which decreases tOC and ucOC (449-451). Due to the link between OC and glucose metabolism, one hypothesis is that bisphosphonate treatment may also affect glucose metabolism. Some observational studies report that adults treated with antiresorptives have a decreased risk of developing T2D (452-456) but not all studies support this. Data obtained from three RCTs including osteoporotic postmenopausal women reported that treatment with antiresorptives (ALN, zoledronic acid and denosumab, DMAb) did not affect differences in fasting glucose or risk for T2D (457) but these trials did not present ucOC data. Similarly, osteoporotic patients treated with risedronate exhibited decreased levels of tOC and ucOC, but this was not associated with changes in glucose metabolism. Similar findings were reported following DMAb treatment in postmenopausal osteoporotic women without diabetes (458, 459) or those with pre-diabetes or T2D (460). In a different study, DMAb reduced ucOC in osteoporotic postmenopausal women (461) and in osteoporotic patients switching from TPTD to DMAb (462), but glucose metabolism was not measured.

In women with hypoparathyroidism, parathyroid hormone (PTH) treatment was reported to increase tOC levels (463) or increase both tOC and ucOC (464). In osteoporotic postmenopausal two studies reported that PTH increased tOC and ucOC levels (465, 466). However, these studies delivering PTH treatment give conflicting results regarding the relationship between the change in OC with glucose metabolism.

For instance, PTH treatment in postmenopausal osteoporotic non-diabetic women increased tOC and ucOC and this was related to decreased BGL (466) but most studies report no link (463-465) or did not measure metabolic outcomes (463).

Conflicting results amongst the studies mentioned above reporting tOC and ucOC could also be explained by several underlying factors i.e. age, sex, clinical characteristics, medications used or menopausal status amongst other factors. Notably, throughout the literature there is also a large variation in the different assays used to assess and measure tOC and ucOC (see assay methods in *Table 1*). Currently, there is no optimal method for the measurement of ucOC. Commonly, a hydroxyapatite (HAP) binding method proposed by Gundberg and colleagues (467) or a direct determination for Glu-OC by an ELISA specific for fully uncarboxylated OC (from Takara) is used, each with limitations. The HAP binding method is based on the lower affinity of ucOC to the HAP compared to fully carboxylated OC. The method is complex and levels are highly dependent on technical details such as antibodies used, specific binding capacity of the HAP, amount of apatite used, or ELISA used. This method does allow the expression of ucOC as a percentage of tOC (ucOC% or ucOC/tOC), as ucOC is measured on the same sample before incubation with HAP after measuring tOC. This may be more clinically informative. There is an available combination kit that recognizes Gla-OC (carboxylated OC) and uncarboxylated OC but neither of these kits recognises undercarboxylated OC. There are some instances where ELISAs can report ucOC levels higher than those of tOC, which may suggest non-specific binding and an overestimation of ucOC. Altogether, as a result of different methods that attempt to measure ucOC, it can be difficult to interpret results and compare study findings. Elucidating the direct effects of ucOC on glucose regulation is essential to our understanding of the function of this bone hormone in general, as it could have clinical implications in identifying new mechanistic targets and treatment avenues for metabolic conditions such as T2D.

2.2.2.1.5 Direct effect of ucOC on muscle glucose metabolism

Skeletal muscle plays a key role in whole-body glucose disposal and energy regulation (97) and therefore is a likely target tissue for ucOC. However, exploring skeletal muscle glucose metabolism and the direct effects of OC in humans is difficult. As such in recent years, mechanistic studies that examine the direct effect, or lack of

effect, of ucOC on muscle have been conducted using a combination of *in vivo*, *ex vivo* and *in vitro* studies (***Table 2.4***).

One of the first studies providing evidence for the direct effect of ucOC on whole-body glucose regulation in rodents, outside the Karsenty group, was published by Speranza et al. (468). They reported that OC plays a critical role in the commonly reported dysregulation of energy homeostasis caused by glucocorticoid use (468). Additionally, they demonstrated that corticosterone (CS) treatment suppresses ucOC by more than 90% in wild-type (WT) mice and this was accompanied by the development of insulin resistance and impaired glucose tolerance. However, when they treated transgenic mice with a specific osteoblast-targeted disruption of glucocorticoid signalling (Col2.3-11bHSD2 Tg mice), these mice had normal circulating levels of ucOC and were protected from the development of insulin resistance, glucose tolerance and abnormal weight gain (468).

As *in vivo* exercise can increase ucOC levels, which in turn is related to increased insulin sensitivity (30), we examined whether ucOC treatment can increase the insulin-sensitizing effects of exercise using an *ex vivo* model of skeletal muscle contraction followed by ucOC treatment (469). We reported that ucOC enhanced the insulin-sensitizing effects of muscle contraction in glycolytic muscle by 14% (extensor digitorum longus, EDL), yet in contrast to our hypothesis, we did not observe changes in basal insulin sensitivity following ucOC treatment. We and others reported that in C2C12 myotubes, ucOC enhances insulin-stimulated glucose uptake compared to insulin stimulation alone (469, 470). As such, it is possible that methodological limitations in our previous study, which used intact muscle, affected the results as only the outer muscle was exposed to ucOC. In follow-up studies, muscles were cut longitudinally to enhance ucOC exposure. We then observed that ucOC (at physiological levels) alone increased muscle glucose uptake in mice EDL and soleus (471), as well as with insulin stimulation, in a muscle specific manner (472). We also demonstrated that ucOC treatment at similar levels can alleviate insulin resistance induced by corticosterone in both glycolytic and oxidative muscles (473). Treatment with exogenous ucOC was shown to induce myoblast proliferation of C2C12 cells *in vitro* via PI3K/Akt and p38 MAPK pathway, and myogenic differentiation involving GPRC6A-ERK1/2 signalling (43). In that study, the inhibition of Akt (wortmannin) and P38 MAPK phosphorylation (SB203580) inhibited (decreased) the effect of ucOC on cell proliferation and the inhibition of ERK 1/2 phosphorylation (U0126) decreased C2C12 cell differentiation. Furthermore, ucOC

treatment increased GPRC6A expression in C2C12 myotubes when silenced (GPRC6A siRNA) activation of Akt, P38 MAPK and ERK 1/2 phosphorylation was inhibited and cell proliferation and differentiation was decreased. Some studies reported that treatment with recombinant ucOC has limited effects on basal or insulin-stimulated glucose uptake and insulin signalling activity, as well as on glycolysis in mouse muscle tissue or cultured myotubes (474-477). One paper even reported that ucOC treatment blunted insulin-stimulated glucose uptake in C2C12 myotubes (474). ***Table 2.4*** summarises the studies of the direct effects of ucOC on glucose metabolism.

Table 2.4 Direct effects of ucOC on glucose metabolism

	Author, year	Experimental Overview	Effect on metabolic function	Potential mechanisms
Genetically modified animals (excluding OC KO)	**Jørgensen**, 2019 (478)	GPRC6A KO mice CS treatment	**Vehicle-treated KO compared to WT:** ↔ basal BGL, INS, IS Similar ↑ in body composition **CS-treated KO compared to WT**: Similar ↑ in body composition and INS Similar ↓ in muscle mass, IS, and basal BGL	**Vehicle-treated KO compared to WT:** ↓ OC ↓ OC mRNA expression **CS-treated KO compared to WT**: Similar ↓ in OC Similar ↓ in OC mRNA expression
	Mao, 2021(479)	LRP1 endothelial cell (EC)-specific inducible knockout mice (eKO) OC treatment (injection 150 μg/kg)	**LRP1 depleted ECs:** ↑ IS ↑ glucose uptake (muscle and white adipose tissue) **LRP1 depleted ECs in HFD mice:** ↑ IS ↑ glucose uptake (muscle and white adipose tissue) ↓ weight gain, blood insulin, glucose, TG, FFA, and HOMA-IR **LRP1 depleted ECs in diabetic mice:** ↑ measures of glucose homeostasis (attenuated with OCN AAV depletion)	**LRP1 depleted ECs:** ↑ serum OC and ucOC ↑ OC mRNA expression ↑ FoxO1 nuclear export **OC treatment:** ↑ pIRS1, pAKT and pGSK3β (muscle and liver), and GLUT4 translocation (muscle) **HFD model:** ↓ blood OC and ucOC (attenuated with LRP1 eKO) **STZ-induced diabetes model:** ↓ blood OC and ucOC (attenuated with LRP1 eKO)

	Author, year	Experimental Overview	Effect on metabolic function	Potential mechanisms
			Daily injection of OC (2 w) in mice with T1DM: ↔ blood INS ↓ BGL	
	Rached, 2010 (480)	$Foxo1_{Osb}^{-/-}$ mice	***FoxO1*$_{Osb}^{-/-}$ compared to WT:** ↓ BGL ↑ insulin ↑ islet number and size, and β cell mass and proliferation ↑ glucose tolerance ↑ IS ↑ glucose disposal ↔ body weight ↓ fat pad ↑ energy expenditure ***FoxO1*$_{Osb}^{-/-}$ mice lacking a single osteocalcin allele:** Metabolic phenotype reversed. ***FoxO1*$_{Osb}^{-/-}$ mice are protected from HFD-induced obesity and insulin resistance.**	***FoxO1*$_{Osb}^{-/-}$ compared to WT:** ↑ expression and circulating OC & ucOC ↑ *Ppargc1a*, *Nrfl* and *Mcad* gene expression in muscle. ↑ *Foxa2*, ↓ *G6Pase* and *Pck1* gene expression and ↓ fat content in liver ↑ expression & circulating levels of adiponectin. ↑ acyl-COA oxidase, Ppara and Ucp2 in muscle. ↔ resistin or leptin
	Smajilovic, 2013 (481)	GPRC6A KO mice	**GPRC6A KO compared to WT:** ↔ basal BGL, INS ↔ glucose and insulin tolerance ↔ body fat %	None

	Author, year	Experimental Overview	Effect on metabolic function	Potential mechanisms
			↔ l-arginine induced insulin secretion	
	Yoshizawa, 2009 (482)	Atf4$^{-/-}$ mice ATF4$_{Osb}^{-/-}$ ATF4$_{Osb}$ overexpression	**ATF4$^{-/-}$ mice compared to WT:** ↑ β cell area and proliferation ↑ glucose tolerance ↑ insulin tolerance ↓ fat pads and blood glucose ↑ INS and INS secretion ↓ gluconeogenesis ↑ glycolysis **Osteoblast specific ATF4$_{Osb}^{-/-}$ similar metabolic abnormalities as ATF4$^{-/-}$** **Osteoblast ATF4 overexpression opposite metabolic response to ATF4$_{Osb}^{-/-}$**	**ATF4$^{-/-}$ mice (including ATF4$_{Osb}^{-/-}$) compared to WT:** ↑ circulating ucOC (ATF4$_{Osb}^{-/-}$) ↓ expression Pck1, G6pase and Pdk4 in liver ↑ Gck and Foxa2 in liver ↑ pAkt and GSK-3β in liver ↑ basal Mcad and pAkt in muscle ↑ Pparg in fat. **Osteoblast ATF4 overexpression compared to WT:** Opposite metabolic abnormalities to ATF4$^{-/-}$ ↓ circulating ucOC
	Zhang, 2020 (483)	KKAy mice ucOC treatment (3, 30 ng/g per day, ig, orally, 4 w)	↓ fasted and non-fasted blood glucose ↑ glucose tolerance ↓ fasting plasma insulin ↓ HOMA-IR ↓ hepatocyte lipidosis ↓ dyslipidemia	**ucOC treated KKAy mice:** ↑ ucOC ↑ Insulin stimulated pIRβ, pAKt, pFoxo1 and pGSK3β in liver. ↑ protein content of CD36 in liver ↓ protein content of SREBP1c, ACC and FAS in liver ↓ expression and content of MCAT in liver
ucOC/drug administration *in vivo*	**Brennan-Speranza,** 2012 (468)	Col2.3-11bHSD2 Tg mice (Overexpress 11bHSD2 in	**all mice treated with CS** ↓ BW	

	Author, year	Experimental Overview	Effect on metabolic function	Potential mechanisms
		osteoblasts resulting in no/reduced corticosterone activity in cells) CS treatment (1.5 mg CS (slow-release pellet, p/week), or placebo, for 28 days	**WT mice treated with CS** vs WT placebo ↑ BW in treated WT ↑ body fat mass ↑ TGLs ; ↑ Chol IR; glucose intolerant ↓↓ tOC and ucOC **Tg mice treated with CS vs TG placebo:** No diff in BW or fat mass No diff in TGL; ↑ Chol Glucose tolerant ↓ tOC (> CS-treated WT mice) ↔ ucOC	**GC supresses OC expression in osteoblasts:** Attenuated reduction in OC levels in TG mic correlate with protection again CS-induced metabolic dysfunction. **Muscle & liver**: no diff in *Gilz* and *Fkbp5* by GC treatment in WT and Tg mice
	Dou, 2014 (484)	ApoE-/- mice HFD OC treatment (daily, 30 ng/g 12 w)	**OC treated chow mice:** ↓ fasting blood glucose ↓ TC and LDL-C ↔ glucose tolerance ↔ insulin tolerance ↔ mean, systolic and diastolic BP ↔ ACh-stimulated EDR **OC treated HFD mice:** ↓ fasting blood glucose ↓ body weight ↓ TC, TG and LDL-C	**OC treated chow mice:** ↔ TNF-α, IL-1 α, IL-12 p70 and IL-12 p40 ↑ pPI3K, Akt and eNOS **OC treated HFD mice:** ↓ TNF-α, IL-1 α, IL-12 p70 and IL-12 p40 ↑ pPI3K, pAkt and peNOS

	Author, year	Experimental Overview	Effect on metabolic function	Potential mechanisms
			↑ glucose tolerance ↑ insulin tolerance ↔ SBP ↓ mean and diastolic BP ↑ ACh-stimulated EDR	
	Guedes, 2018 (485)	Obese mice ucOC treatment (mini pump, 3 ng/h, 4w)	**ucOC treated obese mice:** ↑ insulin sensitivity ↓ white adipose tissue ↔ body weight ↔ plasma insulin ↔ plasma glucose	**ucOC treated obese mice:** ↑ plasma ucOC ↔ GLUT4 protein and Slc2a4 gene expression in skeletal muscle ↑ GLUT4 protein, Slc2a4 gene expression, and pAkt in white adipose tissue ↔ Adipoq gene expression in white adipose tissue ↓ Tnf, Il-1b, Il-6, Ccl2, Casp1, and Nlrp3 gene expression in white adipose tissue ↓ Opg and Ptprv gene expression in bone tissue ↔ GLUT4 protein and Slc2a4 gene expression in bone tissue ↑ pAkt in bone tissue
	Gupte, 2014 (476)	*Ldlr*$^{-/-}$ mice WHFD	**ucOC treated chow mice:** ↔ insulin tolerance ↔ body weight ↔ body fat	**ucOC treated chow mice:** ↔ insulin stimulated pAkt in muscle ↔ plasma AST

	Author, year	Experimental Overview	Effect on metabolic function	Potential mechanisms
		ucOC treatment (mini pump, 4.5 ng/h, 12 w)	↓ liver fat content ↔ liver histology ↔ triglycerides ↔ cholesterol ↔ phospholipids **ucOC treated WHFD mice:** ↑ insulin tolerance ↔ body weight ↔ body fat ↓ liver fat content ↔ triglycerides ↔ cholesterol ↔ phospholipids ↓ pathological changes of NASH	**ucOC treated WHFD mice:** ↑ insulin stimulated pAkt in muscle ↔ insulin stimulated pAkt in liver ↓ plasma AST ↓ Cd68, F4/80 and Cd74 gene expression ↔ MCP1, TNF, Nlrp3, Ciita and adiponectin gene expression in white adipose tissue ↓ Cd68, Spp1, and Il1b Col1a2 and Col4a1 in liver.
	Huang, 2017 (486)	STZ-induced diabetes HFD OC treatment (injection, 30 ng/g, 12 w)	**OC treated control rats:** ↓ fasting blood glucose ↑ fasting insulin ↔ glucose tolerance ↑ IPGTT serum insulin ↓ serum TC and LDL-C ↔ serum TG and HDL-C ↓ body weight and abdominal fat mass ↔ SBP, DBP, PP, MAP, HR, and PWV **OC treated rats with diabetes:**	None investigated

	Author, year	Experimental Overview	Effect on metabolic function	Potential mechanisms
			↓ fasting blood glucose ↑ fasting insulin ↑ glucose tolerance ↑ IPGTT serum insulin ↓ serum TC, TG, and LDL-C ↔ serum HDL-C ↑ body weight ↓ abdominal fat mass ↔ SBP, DBP, PP, MAP and HR ↓ PWV	
	Pandey. 2020 (487)	Pregnant & lactating Wistar rats HFD ALN treatment (100 or 200 µg/kg BW, 2 x p/wk, 6w) Warfarin treatment (injections 0.25 mg/kg BW daily, 3d)	**ALN treated rats** ↓ insulin tolerance **Warfarin treated rats** ↑ insulin tolerance	**ALN treated rats** ↓ serum ucOC ↓ Glut4 content in muscle **Warfarin treated rats** ↑ serum ucOC ↑ Glut4 content in muscle

	Author, year	Experimental Overview	Effect on metabolic function	Potential mechanisms
	Parker, 2018 (488)	Healthy men Acute exercise Insulin stimulation Acute glucocorticoid treatment (GC) (20 mg prednisolone)	**GC treatment**: ↑ FGL and INS ↑ HOMA-IR ↓ post-exercise insulin sensitivity	**GC treatment**: ↓ serum ucOC ↓ GPRC6A content in muscle ↑ basal pAkt, pAS160, pIRS1 ↓ insulin stimulated pmTOR, pAkt, pAS160, pIRS1 ↓ basal and post-ex serum IL-6
	Sabek, 2015 (489)	Nonobese diabetic-severe combined immunodeficiency (NOD-scid) mouse model for in vivo function testing of grafted human islets D-OC treatment (Mini pump, 4.5-ng/h, 30 days)	**OC treated mice:** ↑ human INS and C-peptide secretion	**OC treated mice:** ↑ β-cell proliferation (insulin/glucagon and Ki67 staining)
	Smajilovic, 2013 (481)	GPRC6A KO mice Intravenous injection L-arginine	**Insulin response to L-arginine** ↔ Insulin secretion between KO and WT in fasting mice of mice fed libitum Insulin release max at 1min post, returned to basal at 5min in KO and WT **Insulin release after oral L-arginine or D-glucose**	None measured

	Author, year	Experimental Overview	Effect on metabolic function	Potential mechanisms
			↑ insulin concentration (↔ KO vs WT)	
	Zhou, 2013 (477)	C57BL/6J mice HFD ucOC treatment (mini pump, 3 ng/h implant, 28 d)	**ucOC treated ND mice.** ↔ BW ↓ fat pad ↑ insulin ↑ insulin tolerance ↑ glucose tolerance ↓ serum TGL and FFA ↑ energy expenditure **ucOC treated HFD mice.** ↓ BW and fat pad ↓ insulin ↑ insulin tolerance ↑ glucose tolerance ↓ serum TGL and FFA ↑ energy expenditure	**ucOC treated ND mice.** ↑ circulating OC ↑ Foxa2 in liver ↓ Pepck in liver ↑ Pgc1α and Ucp1 in adipose tissue ↑ Nrf1 and Mcad in skeletal muscle ↔ mitochondria number, area and size in liver, adipose and skeletal muscle ↔ liver weight ↔ Tnfα expression ↔ pERK, peIF2α, p-IRE-1α and ATF6β/c-Jun in adipose tissue, liver and skeletal muscle. **ucOC treated HFD mice.** ↑ circulating OC ↑ Foxa2 in liver ↓ Pepck in liver ↑ Pgc1α and Ucp1 in adipose tissue ↑ Nrf1 and Mcad in skeletal muscle ↑ mitochondria number, area and size in liver, adipose and skeletal muscle ↓ liver weight ↓ Tnfα expression ↓ pERK, peIF2α, p-IRE-1α and ATF6β/c-Jun in adipose tissue, liver and skeletal muscle.

	Author, year	Experimental Overview	Effect on metabolic function	Potential mechanisms
	Zhou, 2016 (475)	C57BL/6J mice HFD ucOC treatment (30 ng g–1 BW, 8w)	**OC treated ND mice.** ↓ body weight ↓ fat-pad weight ↓ serum TGL ↓ FFA ↓ blood glucose ↓ blood insulin ↑ glucose tolerance ↑ insulin tolerance ↑ energy expenditure **OC treated HFD mice.** ↓ body weight ↓ fat-pad weight ↓ serum TGL ↓ FFA ↓ blood glucose ↑ blood insulin ↑ glucose tolerance ↑ insulin tolerance ↑ energy expenditure	**OC treated ND mice.** ↑ serum OC ↑ Pgc1α and Ucp1 in adipose tissue ↑ Pgc1α and Mcad in skeletal muscle ↔ mitochondria number and area in adipose tissue and skeletal muscle ↔ Atg7, p62 and LC3-II in adipose tissue and skeletal muscle ↔ autophagysomes number in adipose tissue and skeletal muscle **OC treated HFD mice.** ↑ serum OC ↑ Pgc1α and Ucp1 in adipose tissue ↑ Pgc1α and Mcad in skeletal muscle ↑ mitochondria number and area in adipose tissue and skeletal muscle ↓ Atg7 and LC3-II in adipose tissue and skeletal muscle ↑ p62 in adipose tissue ↓ autophagysomes number in adipose tissue and skeletal muscle
ucOC treatment in *ex vivo* muscles	**Levinger**, 2016 (490)	Eight-week-old male C57BL/6J mice	**Contraction alone** ↔ muscle GU vs rest **Insulin- in paired resting EDL muscles**	**Protein expression in mouse muscle sections** GPRC6A expression **Insulin- in paired resting EDL muscles**

	Author, year	Experimental Overview	Effect on metabolic function	Potential mechanisms
		ucOC treatment in EDL muscle (10 ng/ml, in the presence or absence INS, 60 μU/ml)	↑ muscle GU **Insulin post contraction** ↑ muscle GU vs contraction alone **Contraction + ucOC treatment+ insulin** ↑ muscle GU vs contraction+insulin **ucOC treatment alone** ↔ muscle GU from baseline ↔ resting insulin sensivity **ucOC post-ex vivo contraction (no insulin)** ↔muscle GU vs contraction alone	↑ p-Akt, p-akt/tAkt, p-AS160 vs controls ↔ tAkt, AS160 **Insulin post contraction** ↑ p-Akt, p-akt/tAkt, p-AS160, p-AS160/AS160 vs contraction alone **Contraction + ucOC treatment + INS** ↑ P-AS160, Akt vs contraction + insulin **ucOC post-ex vivo contraction (no insulin)** ↔ p-Akt, p-AS160 vs contraction alone
	Lin, 2017 (471)	Eight-week-old male C57BL/6J mice ucOC treatment in mouse EDL and soleus muscle splits (0, 0.3, 3, 10, 30 ng/mL 1.5 h)	**ucOC treatment in EDL muscle splits** ↑ basal GU (10 and 30 ng/mL) **ucOC treatment in soleus muscle splits** ↑ basal GU (0.3 and 30 ng/mL)	**ucOC treatment in EDL muscle splits** *ucOC at 30ng/mL*: ↑ pMTOR , p-mTOR/tmTOR ratio, tAS160, p-ERK2 *ucOC 3ng/mL:* ↑ p-AS160, p-AS160/tAS160 ratio *ucOC 3 and 30 ng/mL:* ↔ AMPKα

	Author, year	Experimental Overview	Effect on metabolic function	Potential mechanisms
				ucOC treatment in soleus muscle splits *ucOC 3ng/mL:* ↑ p-AS160, p-AS160/tAS160 ratio *ucOC 3 and 30 ng/mL:* ↑ p-ERK2 ↔ AMPKα *ucOC 30ng/mL:* ↑ tAMPKα
	Lin, 2018 (472)	eight-week-old male C57BL/6J mice ucOC treatment in EDL and soleus muscle splits (0, 0.3, 3, 10, 30 ng/mL 1 h)	**ucOC treatment in EDL muscle splits** ↔ IS GU **ucOC treatment in soleus muscle splits** ↑ in basal ↑ IS GU	**ucOC treatment in EDL muscle splits** *ucOC 30ng/mL:* ↔p-Akt, p-AS160. ↑ tGlut4 **ucOC treatment in soleus muscle splits** *ucOC 30ng/mL* ↑ p-AS160 ↔ Glut4
	Lin, 2019 (473)	Eight-week-old male C57BL/6 J mice implanted CS slow-release pellets 3 days	**CS-treated mice vs placebo mice** ↓ OC ↓ ucOC ↑ INS ↑ fasting BGL ↑ BGL during ITT (60, 90 min)	**Placebo EDL muscle** ***INS alone:*** ↑ p-mTOR/tMTOR ratio, p-Akt, p-Akt/tAkt ratio ***ucOC treatment:*** ↑ p-mTOR, p-AS160

	Author, year	Experimental Overview	Effect on metabolic function	Potential mechanisms
		ucOC treatment (30 ng/mL 1 h)	**INS treatment EDL muscle** ↑ GU in placebo mice ↔ GU CS-treated mice **ucOC treatment** ↑ GU EDL and soleus in placebo mice ↔ GU EDL and soleus in CS-treated mice ↑ IS GU EDL CS-treated mice ↔ IS GU soleus CS-treated mice ↔ IS GU EDL or soleus placebo mice	***ucOC+INS vs INS alone*** ↑ GPRC6A, p-PKCζ/λ **CS-treated EDL muscle** ***INS alone:*** ↑ p-Akt, p-Akt/tAkt ratio ***ucOC+INS vs INS alone*** ↑ p-mTOR, p-Akt, p-AS160/tAS160 ratio, p-ERK2, p-ERK2/tERK ratio ↓ AS160 ↔tmTOR, p-mTOR/tmTOR ratio, pAS160, tERK2, p-AMPKa, tAMPKa, p-AMPKa/tAMPKa ratio, p-PKC **Placebo soleus muscle** ***INS alone:*** ↑ p-Akt, p-Akt/tAkt ratio ***ucOC treatment:*** ↑ p-mTOR, p-mTOR/tMTOR ratio, p-AS160, p-PKC ***ucOC+INS vs INS alone*** ↑ p-mTOR, p-AS160

	Author, year	Experimental Overview	Effect on metabolic function	Potential mechanisms
				CS-treated soleus muscle ***INS alone:*** ↑ p-Akt, p-Akt/tAkt ratio, p-AMPKa, p-AMPKα/tAMPKα. ***ucOC+INS vs INS alone*** ↑ p-mTOR, p-mTOR/tmTOR ratio, p-AS160, p-AS160/tAS160 ratio, p-PKC ↔tmTOR, tAS160, pAS160/tAS160 ratio, tGPRC6A, p-ERK2, tERK2, p-ERK2/tERK2 ratio, p-PKC
ucOC treatment *in vitro* cell culture	**Guedes**, 2018 (485)	Mouse 3T3-L1 adipocytes ucOC treatment (20 ng/mL, 24 h; pre-treatment: 20 ng/mL 6 h, followed by 20 ng/mL TNF for 18 h)	None measured	**24h ucOC treatment** ↑ expression of *Slc2a4* and GLUT4 ↑ Adipoq expression ↑ AKT phosphorylation after INS **ucOC pre-treatment** ↓ the NFKB subunit p65 activation in TNF-a induced cells ↓ Tnf, Ccl2, Nfkb1 ↔ Adipoq ucOC restored *Slc2a4*/GLUT4 content and ↓ expression of inflammatory genes after TNF-a challenge
	Guo, 2017 (491)	HUVECs	**HUVECs treated with Tunicamycin** *Tun-treated cells* ↓ GU	**HUVECs treated with Tunicamycin** *Tun-treated cells* ↑ protein expression ATF4 and CHOP

	Author, year	Experimental Overview	Effect on metabolic function	Potential mechanisms
		ucOC treatment (5 ng/mL for 4 h), tunicamycin treatment (5 µg/mL for 4 h) insulin stimulation (10 nM for 10 min) wortmannin treatment Akti-1/2 treatment (10 µM for 4 h) Palmitate treatment (500 uM)	**ucOC treatment in tun-treated cells** ↑ GU	↓ p-ERK, p-eLF2α ↓ IS p-IRS-1 tyrosine and p-Akt **ucOC treatment in tun cells vs tun-treated cells**. ucOC alleviated Tun-induced ER stress & improved INS signalling ↓ protein expression ATF4 and CHOP ↓ p-ERK, p-eLF2α ↑ p-AKT, IRS-1 ↑ P13k activity in presence of IR **HUVECs treated with palmitate** *Palm-treated cells- induced IR, ER stress and impaired INS signalling, ucOC reversed these effects* *ucOC treatment in palm cells vs untreated palm cells* ↓ p-ERK, p-eLF2α, ATF4 and CHOP ↓ pY20 and p-Akt
	Hill, 2014 (492)	L6 rat myotubes and adipocytes, ten week old male C57BL/6 J mice and 150–180 g male Wistar rats OC treatment (Adipocytes: 1	**Rat adipocytes treated with cOC** ↑ basal GU in cOC treated (dose-dependent) ↑ IS GU in cOC treated (dose-dependent) ↑ insulin sensitivity in cOC treated	**Rat adipocytes treated with cOC and ucOC** ↓ TNFα secretion in cOC and ucOC treated cOC treatment ↓ IL-6 secretion, ↔ with ucOC. ↔ MCP-1 with cOC or ucOC

	Author, year	Experimental Overview	Effect on metabolic function	Potential mechanisms
		ng/mL cOC and ucOC, 1 h; L6 myotubes: 20ng/mL cOC) Insulin stimulation	**Rat adipocytes treated with cOC vs controls** ↑ basal glucose oxidation ↔ IS glucose oxidation ↔ basal of IS lipogenesis, lipolysis or antilipolysis **Mouse adipocytes treated with cOC and ucOC** ↑ basal GU both cOC and ucOC ↑ IS GU both cOC and ucOC ucOC greater than cOC at ↑ basal GU and ↑ insulin sensitivity **L6 myocytes treated with cOC vs controls** ↑ basal GU and IS GU **Rat adipocytes and whole adipose tissue treated with cOC and ucOC** ↑ Adiponectin secretion	**Whole adipose tissue treated with cOC and ucOC** ↔TNFα, IL-6, and MCP-1 ↑ secretion IL-10
	Idelevich, 2011 (493)	Cultured vascular smooth muscle (MOVAS) and chondrocytes (ATDC5) from murine aorta, cells induced with calcification and	**MOVAS and ATDC5 cells** Express BGP **BGP-overexpressed ATDC5 and MOVAS cells vs controls** ↑ BGP mRNA and protein	**BGP-overexpressed ATDC5 and MOVAS cells vs controls** ↑ Sox9, Runx2 and collaged type X ↑ collagen type II in ATDC5 cells ↓ collagen type II in MOVAS cells ↑ mRNA levels of Glut1 in ATDCF cells

	Author, year	Experimental Overview	Effect on metabolic function	Potential mechanisms
		overexpressed with OC. Treated with purified bone Gla protein (BGP) 24 h post transfection (100 nmol/L diluted in normal ATDC5 or MOVAS medium, for 0, 5, 10, 15, 30, and 60 m)	↑ differentiation and mineralisation (↑ mineral deposits, proteoglycans, ALP) ↑ metabolic cleavage of XTT ↑ densities ↑ GU	↑ mRNA levels of Glut4 in MOVAS cells ↑ glycolysis enzymes- expression PFK1 and PDK1 in both cells ↓ glucogenesis enzymes- mRNA expression phosphoenolpyruvate caboxykinase and glucose-6-phosphatase **WT ATDC5 and MOVAS cells treated with BGP** Exogenous BGP activated insulin signalling pathway ↑ p-IRS1, and p-Akt **Silencing HIF-1α using siRNA with BGP** BGP treatment: ↑ HIF-1α, silencing HIF-1α counteracted this in ATDC% and MOVAS cells **Treatment of siRNA with BGP** In ATDC5 cells, time-course dependent upregulation of mRNA PFK, PDK1, GLUT1, IRS-1, silencing HIF-1α prevented this response In MOVAS cells, same response as ATDC5 cells, except silencing HIF-1α supressed GLUT4, not GLUT1.
	Jung, 2013 (494)	human aortic endothelial cells	**Pre-treatment ucOC (30ng/mL) in HAECS** ↓ LA induced apoptosis in IS-HAECS	**Treatment of ucOC in HAECS vs controls** ↑ p-Akt, 0.5 to 4hrs

	Author, year	Experimental Overview	Effect on metabolic function	Potential mechanisms
		ucOC treatment (0.3–30 ng/mL) linoleic acid (100 µmol/L for 16 h) wortmannin (100 nmol/L for 15 min)		↑ p-Akt post ucOC treatment ≥3 - 30ng/mL ↔ tAKT **HAECs treated with wortmannin** prevented ucOC induced phosphorylated of Akt. **Pre-treatment of ucOC+INS before LA in HAECS** INS: ↑ p-Akt, whereas LA ↓ this IS ↑ p-Akt ucOC+INS restored p-Akt vs controls (pre-treatment wortmannin- blocked this effect) **treated HAECs with ucOC** (0.3 to 30ng/mL) ↑ p-eNOS, addition of wortmannin prevented this ucOC-induced phosphorylation of eNOS ↑ NO levels vs controls **Pre-treatment of ucOC on IS-HAECs** LA-induced ↑ in apoptosis in IS-HAECs was significantly inhibited by ucOC pre-treatment. Pre-treatment with wortmannin abolished this anti-apoptotic effect of ucOC.
	Levinger, 2016 (490)	C2C12 mouse myotubes ucOC treatment (0.3, 3, 10 and 30 ng/ml, 1h)	**C2C12 myotubes treated with ucOC** ↑ IS GU – dose-dependent 10 - 30 ng/mL	**Differentiated C2C12 myotubes** Expression GPRC6A detected ↓ GPRC6A expression in siRNA(siGPRC6A) vs non-transfected, controls

	Author, year	Experimental Overview	Effect on metabolic function	Potential mechanisms
		insulin stimulation		
	Liu, 2017 (43)	C2C12 myoblasts ucOC treatment (doses 0-50ng/ mL). phosphatidylinositol3 -kinase (PI3K) inhibitor wortmannin transfection GPRC6A siRNA	None measured	**ucOC treatment in C2C12 proliferation** ↑ cell proliferation/number (dose dependent) ↑ p-Akt and p-P38 MAPK (10ng/mL, 24 h) myoblasts express GPRC6A protein and mRNA **C2C12 cells pre-treatment with wortmannin** Akt phosphorylation attenuated ↓ cell proliferation **Inhibition of P38 MAPK (SB203580)** Inhibited effect of ucOC of cell proliferation ↔ Akt Wortmannin pre-treatment ↓ P38 MAPK ↔ p-ERK with ucOC treatment between groups C2C12 cell differentiation through p-ERK 1/2 (10ng/mL) **GPRC6A siRNA knockdown** activation of Akt, P38 MAPK, ERK 1/2 inhibited ↓ C2C12 cell proliferation

	Author, year	Experimental Overview	Effect on metabolic function	Potential mechanisms
				ucOC treatment in C2C12 differentiation vs control ↑ myotubes size (larger) ↑ nuclei per myotubes ↑ expression muscle-specific protein MyHC (0.1 to 50 ng/mL) **ucOC treatment 10ng/mL in cell myogenesis** ↔ P13K/Akt and P38 MAPK pathways ↑ p-ERK1/2 ↔ t-ERK **Inhibition of ERK 1/2 (U0126) in C2C12 cells prior to ucOC treatment** ↓ p-ERK ½ and expression MyHC ↔ GPRC6A
	Parry, 2020 (474)	C2C12 mouse myotubes ucOC treatment (1, 10, 100 ng/mL, 72 h).	**ucOC treated C2C12 myotubes** ↔ basal GU all doses supressed IS GU- 10ng/mL ↔ glycolysis all doses	**ucOC treated C2C12 myotubes** ↔ p-IRS-1, p-Akt, and Glut4: 10 ng/mL for 72 h in normal or IR cells induced by hyperinsulinemia
	Sabek, 2015 (489)	human islets in culture OC (0.3-1.0 ng/mL) and D-OC treatment (1.0 to 15 ng/mL)	**D-OC treatment in human islets 7 & 14 d** 7d: 1.0, 4.5 and 15 ng/mL ↑ INS content of glucose-stimulated islets 14 d: 4.5 ng/mL only ↑ INS content	**D-OC treatment in human islets 7 d** SUR1 ↑ in islets cultured with 4.5 ng/mL 1.0 ng/mL: ↑ %β cell content vs controls 1.0 ng/mL: ↓ %α cell content vs controls ↔ PP cells

	Author, year	Experimental Overview	Effect on metabolic function	Potential mechanisms
			OC treatment in human islets 0.3-1.0 ng/mL ↔ INS content vs controls	
	Smajilovic, 2013 (481)	pancreatic islets were isolated from GPRC6A KO and WT mice incubated with 20 mM L-arginine in the presence of 11 mM D-glucose	↔ insulin release between isolated islets from KO or WT	Expression of GPRC6A in islets if WT, absent in KO
	Tsuka, 2015 (470)	C2C12 mouse myotubes ucOC treatment GluOC at (5 ng/ml) with and without INS	**GluOC treatment on IS GU in C2C12 myotubes** INS: GU ↑ dose-dependent manner GluOC: enhanced IS GU	**C2C12 myotubes** GPRC6A presence **GluOC treatment in C2C12 myotubes** ↑ p-ERK dose dependant manner 0.1 to 30 ng/mL H89 (PKA inhibitor) did not suppress GluOC-induced ERK phosphorylation U0126 (MEK inhibitor) suppressed ERK phosphorylation below basal U73122 (phospholipase C inhibitor) ↔ ERK phosphorylation LY294002 (P13K inhibitor) ↔ ERK phosphorylation, but inhibited basal Akt phosphorylation

	Author, year	Experimental Overview	Effect on metabolic function	Potential mechanisms
				GluOC pre-treatment in C2C12 myotubes for 5ng/mL last 20 m or 72 hrs + IS 20 m: ↑ p-Akt and p-ERK (no IS) 72 h: + IS ↑ p-Akt , ↔ p-Tyr 24 h: ↔ IS Akt, ↔ IRβ **GluOC pre-treatment + U0126 72 h in C2C12 myotubes** Inhibition of MEK abolished GluOC-mediated promotion of INS-induced Akt phosphorylation without affecting basal Akt phosphorylation
	Zhang, 2020 (483)	Mouse primary hepatocytes GluOC treatment (0, 3, 30 ng/mL, 24 h) insulin stimulation	**GluOC treatment in hepatocytes** concentration dependent inhibition of hepatic glucose production with and without insulin stimulation	**GluOC treatment on glycogen synthesis in hepatocytes** ↑ IS GSK3β phosphorylation
	Zhou, 2013 (477)	3T3-L1 adipocytes, Fao liver cells, and L6 muscle cells OC treatment (5 ng/mL, 4 h) Insulin stimulation	None measured	**Adipocytes treated with tunicamycin** ↑ p-ERK, elF2α and IRE-1α and expression ATF6β ↓ IS IRS-1 and Akt **OC treatment in adipocytes, liver and muscle cells treated with tunicamycin**

	Author, year	Experimental Overview	Effect on metabolic function	Potential mechanisms
		Tunicamycin Inhibitors: wortmannin, Akti-1/2, U0126, pyrrolidone dithiocarbamate (NF-kB) NF-kB-p65 siRNA transfection XBP-1 siRNA transfection		↓ Phosphorylation of p-ERK, p- elF2α and p-IRE-1α and expression ATF6β compared to tunicamycin alone ↑ IRS-1 and Akt **Cells treated with insulin, tunicamycin and OC with and without inhibitors** ↑ P13K activity and NF-k β-p65-DNA activity (liver cells under ER stress) Addition of wortmannin or akti-1/2 reversed OC effects on NF-k β-p65-DNA activity Addition of U0126 ↔ **Blocking NF-kB (pyrrolidone dithiocarbamate) and NF-kB -p65 siRNA** ↔ OC protective effect on ER stress and impaired INS signalling induced by tunicamycin **XBP-1 siRNA transfection in cells tunicamycin** ER stress induced, insulin signalling impaired Addition of OC treatment suppressed phosphorylation of p-ERK and increased IRS-1
	Zhou, 2016 (475)	Mouse adipocyte 3T3-L1 cells and mouse C2C12	None measured	**Adipocytes and myocytes treated with tunicamycin** ↑ ER stress ↑ p-ERK and elF2α

	Author, year	Experimental Overview	Effect on metabolic function	Potential mechanisms
		Pre-treatment tunicamycin (5 μg ml· 4 h) then 5 ng ml OC for 4 h Palmitate treatment XBP-1 siRNA Autophagy inhibitor (3-methyladenine or Atg7 siRNA) Inhibitors: Akti 1/2, rapamycin, U0126, pyrrolidone dithiocarbamate (NF-kB)		↓ IS tyrosine phosphorylation IRS-1 and Akt ↑ autophagy (↑ Atg7 and LC3-II, ↓ p62) **Adipocytes and myocytes treated with tunicamycin and OC** ↓ p-ERK and eIF2α phosphorylation reversed autophagy (Atg7, p62 and LC3-II) ↑ IS tyrosine phosphorylation IRS-1 and Akt Restored phosphorylation of Akt and mTOR and maintained sensitivity of Akt to insulin **Adipocyte and myocyte treatment with palmitate** OC treatment alleviated autophagy, ER stress, and insulin signalling **Transfection with XBP-1 siRNA and OC treatment XBP -/- cells adipocytes and myocytes** ↓ expression levels of tunicamycin induced Atg7 and LC3-II protein and P-ERK phosphorylation, and ↑ expression p62 and IRS-1 phosphorylation **Transfection 3-methyladenine and Atg7 with OC treatment adipocytes ad myocytes** ↓ 3-methyladenine- and atg7-induced p-ERK phosphorylation and ↑ IRS-1

	Author, year	Experimental Overview	Effect on metabolic function	Potential mechanisms
				Insulin ,tunicamycin and OC treated adipocytes and myocytes with inhibitors Akti 1/2 and rapamycin: nullified protective effect of OC U0126: did not reverse effects of OC on autophagy or ER pyrrolidone dithiocarbamate: reversed protective effects of OC on autophagy, ER stress and insulin signalling

Key: IS insulin stimulation; INS insulin; GLU glucose; OC osteocalcin; ucOC undercarboxylated osteocalcin; BGP bone gla protein; IR insulin resistance; MetS metabolic syndrome; WAT white adipose tissue; GLUT glucose transporter; BGL blood glucose; FGL fasting blood glucose; GPRC6A, G protein-coupled receptor class C group 6 member A

Current studies have also explored the molecular mechanisms by which ucOC directly enhances muscle glucose metabolism (***Figure 2.16***). Some suggest that, in cultured myotubes, ucOC treatment is capable of directly activating insulin-signalling proteins including the insulin receptor (IR), insulin receptor substrate 1 (IRS-1), Protein kinase B (Akt), Akt substrate of 160 kDa (AS160) and glycogen synthase kinase 3 (GSK3), with or without the presence of insulin (469-471, 495, 496). It appears that ucOC can induce activation of AS160 without increasing Akt phosphorylation, indicating that ucOC could also increase muscle glucose uptake in a Akt-independent manner (469, 471, 472). Although the receptors of ucOC in muscle cells are still unclear, GPRC6A has been suggested to be one of the most promising candidates (495). The knockdown of GPRC6A via RNA silencing in cultured muscle cells was found to abrogate the direct effect of ucOC on glucose uptake without insulin stimulation (496). Some downstream targets of ucOC/GPRC6A cascade, including ERK, AMPK, CREB and PKC, have also been suggested to be the mediators between ucOC stimulation and the activation of the insulin signalling pathway (471, 473, 495). However, it is likely that many unknown targets of ucOC remain to be discovered. Therefore, to delineate the underlying mechanisms, future studies with quantitative proteomics and phosphoproteomics are warranted.

Other groups have provided evidence that ucOC improves human β-cell function (489). Mouse kidneys transplanted with human islets and treated *in vivo* with a vehicle control (PBS) or decarboxylated-OC (D-OC, 4.5 ng/h for 30 days post-transplant) augmented production of human insulin and C-peptide (489). In addition, D-OC treatment *in vitro* of human islet in culture at dose ranges of 1 to 15 ng/mL augmented insulin content and enhanced human B-cell proliferation (489). Additionally, the effects of ucOC treatment on metabolic function has been shown in various organs including *in vivo* models of mouse liver (476, 479), adipose tissue (475, 476, 485) as well as *in vitro* in cells including mouse and rat adipocytes (475, 477, 485, 492), hepatocytes (483), human umbilical vein endothelial cells (491), chondrocytes (ATDC5) and vascular smooth muscle cells (MOVAS) (493) and human islets (489) (***Table 2.4***).

To summarise, considerable evidence exists to support the notion that exogenous ucOC has an effect on muscle glucose metabolism. However, not all studies have consistently reported beneficial effects of OC treatment on energy homeostasis. It is possible that the discrepancies are due to a number of confounding factors that may influence data collection and interpretation, as discussed below.

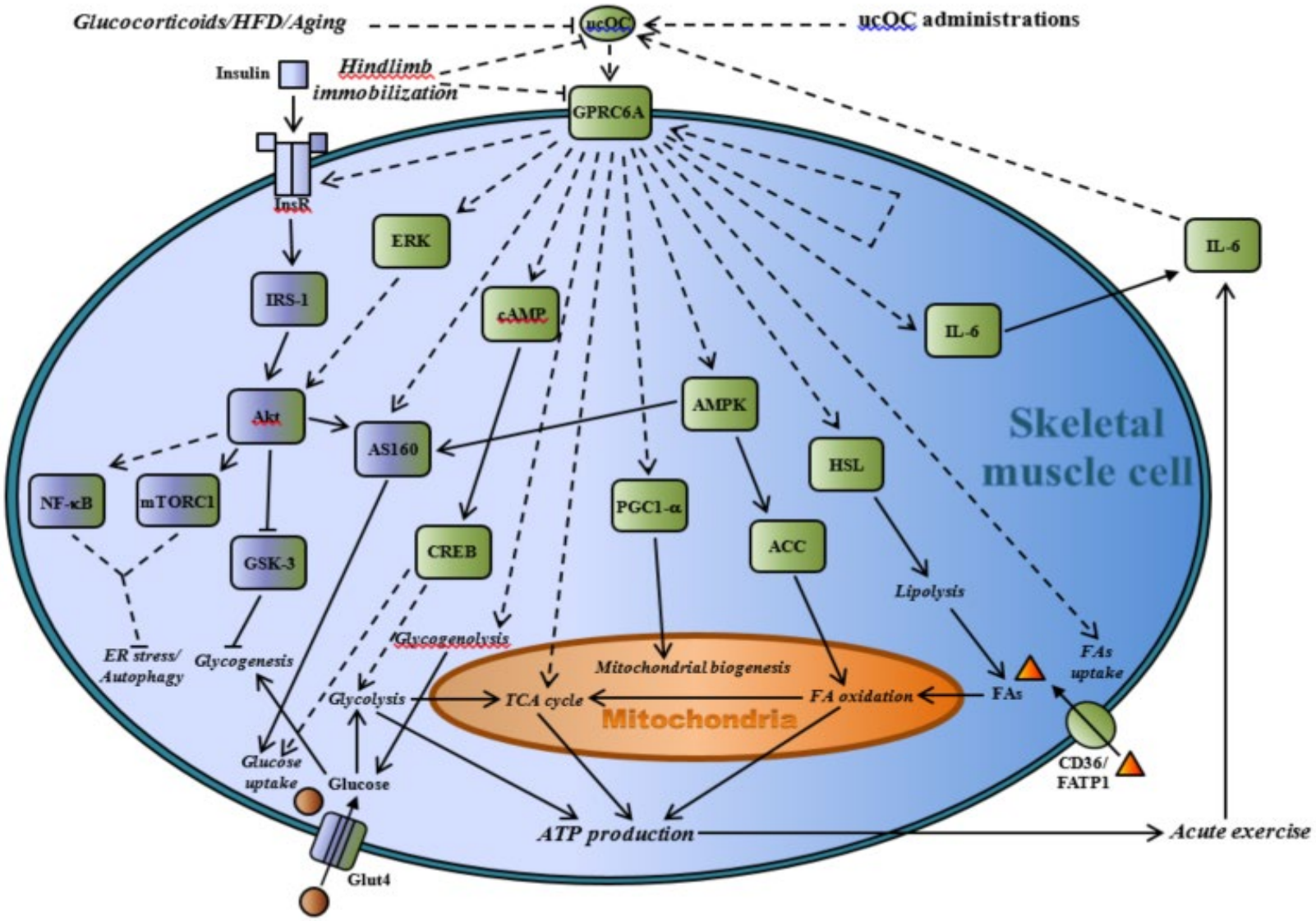
Glucocorticoids/HFD/Aging
ucOC
ucOC administrations
Insulin
Hindlimb immobilization
GPRC6A
InsR
ERK
IRS-1
cAMP
IL-6
IL-6
Akt
AS160
AMPK
Skeletal muscle cell
NF-κB
mTORC1
GSK-3
CREB
PGC1-α
HSL
ACC
Lipolysis
ER stress/ Autophagy
Glycogenesis
Glycogenolysis
Mitochondrial biogenesis
FAs uptake
Glycolysis
TCA cycle
FA oxidation
FAs
Mitochondria
Glucose uptake
Glucose
CD36/ FATP1
ATP production
Acute exercise
Glut4

Figure 2.16 Suggested signalling pathways underlying the effects of ucOC on muscle energy metabolism ucOC regulates muscle energy metabolism via signalling pathways involving GPRC6A as the receptor. The ucOC-induced enhancement of glucose uptake (both non-insulin-stimulated and insulin-stimulated), glycolysis, and insulin-stimulated glycogenesis is mediated via a sophisticated signalling network including GPRC6A, ERK, Akt, AMPK, AS160, GSK-3, the cAMPK/CREB axis, and Glut4. The activity of other downstream targets of Akt such as mTORC1 and NF-κB are also enhanced, attenuating ER stress and autophagy accompanied with insulin resistance. The utilization of other intracellular carbohydrate sources, such as glycogen, is elevated via the ucOC/GPRC6A cascade. Furthermore, ucOC increases FA uptake via enhancing the expression of FA transporters including CD36 and Fatty acid transport protein 1(FATP1), and also enhances lipolysis via enhancing the activity of HSL. In addition, ucOC favors mitochondrial biogenesis and mitochondrial functions in terms of TCA cycle and FA oxidation, via the upregulation of PGC1-α and the AMPK/ACC axis. Lastly, the ucOC/GPRC6A cascade self-amplifies the signalling strength by increasing the expression of GPRC6A. Overall, ucOC-induced enhancement of transport and utilization of nutrients contributes to the production of ATP. During exercise, ucOC levels are considerably increased via the ucOC-IL-6 reciprocal regulation, leading to increased ATP production to fulfil the energy demands of muscle cells. In animals with ucOC administration, ucOC is enhanced, leading to higher levels of energy metabolism and ATP production. On the contrary, in glucocorticoid-treated, HFD-fed and old animals of which ucOC is reduced, energy metabolism and ATP generation are impaired.

Green boxes represent downstream proteins of the ucOC/GPRC6A cascade. Boxes with both blue and green colours represent proteins that are regulated by both proximal insulin signalling cascade and ucOC/GPRC6A cascade.

→ : well-studied activation or enhancement;

⇢ : recently suggested activation or enhancement;

⊣ : recently suggested inhibition;

→ : translocation

2.2.2.1.6 Confounding factors

Differences in genetically modified animals with suppressed or overexpressed circulatory ucOC may contribute to the disparate findings in the literature (***Table 2.4***). It is possible that compensatory mechanisms are activated or the methods used to generate the specific or modified animal may inadvertently lead to several off-target effects. For instance, several studies have suggested that the CRISPR/Cas9 system, which has been used in two studies to delete *Oc gene* in rats and mice (317, 324), can induce a substantial amount of off-target mutagenesis, generating undesired mutations at random sites and thus impacting precise gene modification (497, 498). Furthermore, the differences may also be attributed to the variances of mouse genetic background, as it has been shown that considerable strain-dependent differences in glucose metabolism exist in mouse strains frequently used for genetic manipulation (499). Whether such differences are the source of discrepancies between studies is not clear but it highlights that caution must be taken when comparing data from different studies.

The discordance in current findings may also have resulted from several common confounding factors in experimental settings, such as the source of animals, administration techniques, the type and source of cell-lines, as well as treatment dose, duration and the muscle conditions (intact or split, resting or following contraction). One particular factor that needs emphasis is the source of recombinant ucOC used in these studies. Currently studies report the usage of several types of house-made or commercial ucOC peptides but the variance of biological activity and the magnitude of effect among these peptides is largely unknown.

Another potential source of differences is the use of ucOC under normal conditions versus the use under pathological conditions. There is a substantial amount of evidence that ucOC is capable of improving insulin action in insulin resistant muscle, without altering basal glucose handling and signalling activity, in both *in vivo* and *in vitro* models (473, 475-477). Although current results are promising, the magnitude of the effect as well as the clinical relevance is still not clear.

2.2.2.1.7 Conclusion

In conclusion, there is corroborative evidence from different independent research groups to demonstrate that ucOC acts as a hormone and it has the capacity to increase muscle insulin sensitivity and glucose regulation in mice. As such, we oppose the

unequivocal conclusion of Manogalas' perspective that "osteocalcin promotes bone mineralization but is not a hormone" (325). However, we agree that there are conflicting results and the effect of ucOC is varied and depends on many factors and conditions. To enable the field to move forward, greater transparency and sharing of knowledge and animal models are needed to identify the reasons for the different reports.

2.2.2.2 *Interaction between osteocalcin and muscle function*

There is a substantial amount of evidence that ucOC has systemic effects outside of the bone in glucose regulation and in the last decade increasing evidence supports the potential role of ucOC in bone-muscle crosstalk (***Figure 2.17***).

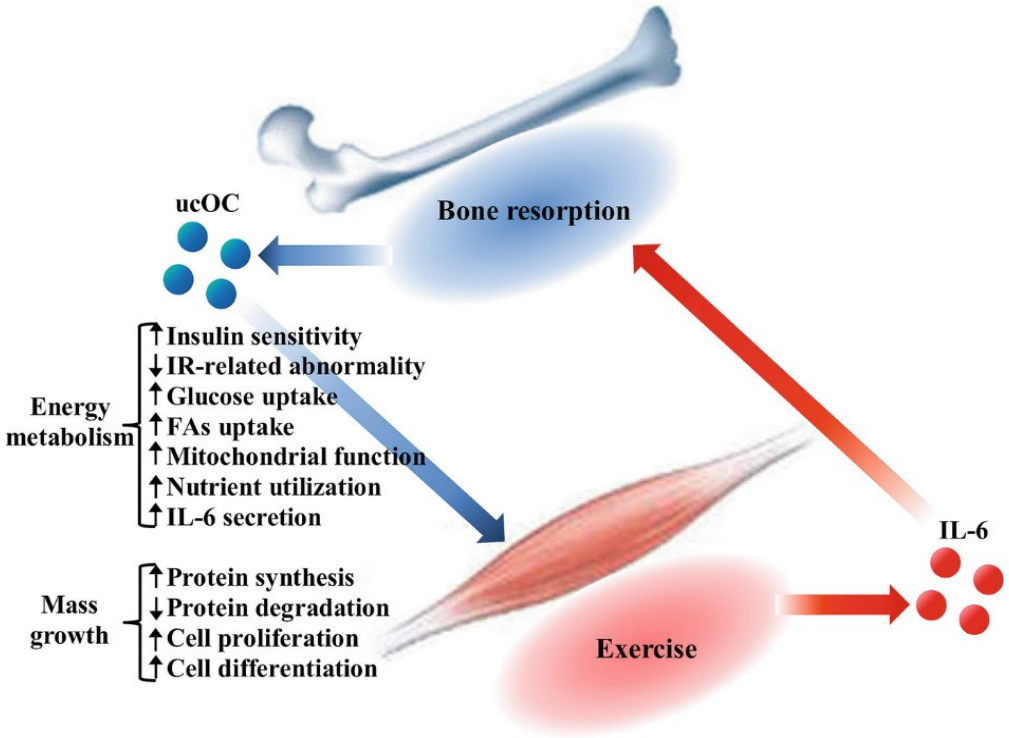

Fig 2.17 Suggested effects of ucOC on skeletal muscle in glucose regulation and muscle mass maintenance. Figure sourced from Lin et al. (500).

There is some emerging evidence, mainly in rodents and pre-clinical *in vitro* studies, suggesting that ucOC may also be involved in the maintenance of muscle mass and function (***Table 2.5***) (12, 16, 27, 43) but this link has not been demonstrated in all studies (313).

Table 2.5 Animal and pre-clinical studies linking ucOC with muscle mass maintenance and strength

Studies Author (year)	**Experimental design**	**ucOC effect on muscle function**
Lin, (2016) (27)	Fischer (F344) rats hindlimb immobilisation	↑ **ucOC** related to ↑ muscle **mass** and **strength** (EDL and soleus)
Liu, (2017) (43)	C2C12 myoblasts *in vitro* ucOC treatment	↑ **proliferation** and **differentiation**
Mera, (2016b) (16)	$Oc^{-/-}$ mice	↓ muscle **volume** and **mass**
	ucOC administration *in vivo* via osmotic pumps	**Improved** muscle **volume** and **mass**
	Primary mice myotubes *in vitro* ucOC treatment	↑ **protein synthesis**
Moriishi, (2020), (313)	$Oc^{-/-}$ mice	↔ muscle mass
Shen, (2015) (12)	$Cx43_{osb/osc}{}^{-/-}$ mice	↓ muscle **volume, mass**, and **strength**
	$Cx43_{osb/osc}{}^{-/-}$ mice- exogenous ucOC treatment via injection	**Normalized** muscle **volume, mass**, and **strength**
	C2C12 myotubes *in vitro* ucOC treatment	↑ myotube **formation**

2.2.2.2.1 Direct evidence for relationship between ucOC and muscle function: animal and in vitro models

At least one study reported that ucOC is necessary for physiological adaptions following exercise (322). The authors reported that exogenous ucOC treatment increases exercise capacity of older mice. The proposed mechanism was that OC signalling in myofibers favours exercise-induced secretion of IL-6, an important regulator of muscle adaption to exercise (322).

In a follow-up study, the Karsenty group reported that mice lacking OC (Ocn -/-) were characterised by lower muscle mass and volume compared to their wild-type (WT) littermates but muscle strength was not different (16). In addition, mice lacking GPRC6A in myofibers (Gprc6a -/-), the putative receptor for OC, had lower muscle weight

compared to WT littermates, supporting the role of GPRC6A as the receptor in myofibers for OC and a role for OC muscle mass regulation. Furthermore, *in vitro* treatment with ucOC in myotubes was partly related to improved muscle mass regulation via the stimulation of an mTOR target related to muscle protein synthesis. Moreover, exogenous administration with ucOC increased muscle mass of older mice (16). Altogether, these data are supportive of a role for ucOC in muscle mass regulation, but provide no evidence for a more functional role in muscle strength.

In addition, mice with Connexin43 deletion in osteoblasts (cKO) have lower circulating cOC and ucOC and lower muscle mass and grip strength compared to their WT littermates (12). Exogenous treatment of ucOC in these cKO mice increased muscle cross-sectional area (extensor digitorum longus) and grip strength (12). Rats with disuse atrophy induced by hind-limb immobilisation, had reduced serum ucOC and this was related to reduced muscle mass and strength in EDL and soleus muscles (27). *In vitro* studies also suggest that ucOC may be involved in promoting muscle growth. C2C12 myotubes treated with ucOC *in vitro* increased myotube formation (12). Cultured C2C12 myoblasts treated with ucOC increased myoblast proliferation via stimulation of the P13/Akt and p38 MAPK pathways and differentiation and in part via the GPRC6A signalling (43). These beneficial effects on myotube formation are important for muscle growth. Yet, more recently, OC-deficient mice (OC-/-) that were created with a different genetic background to the mice from the Karsenty group were shown to have normal muscle mass and glucose metabolism, leading this group to conclude that OC has no role in muscle regulation or mass (313). Discrepancies between studies utilising KO animals could be related to differences in the genetic backgrounds of the models used. Altogether, however, the majority of these animal studies and pre-clinical studies report some role of ucOC in muscle mass regulation and possibly strength.

2.2.2.2.2 Observational evidence: human studies

In humans, there is some observational evidence demonstrating that ucOC (33, 35, 36), tOC (501-504) or the ucOC/tOC ratio (26, 34) are related to muscle health (***Table 2.6***) but the data are contradictory. Some support the view that higher ucOC or a higher ucOC/tOC ratio is related to better muscle health (higher mass, strength) in postmenopausal women (26, 35) and osteoporotic postmenopausal women with previous fractures (33). Yet, even in the studies supporting this link the findings are unclear. For

instance, in older women, a higher ucOC/tOC ratio, but not the absolute value of ucOC or tOC, was related to higher muscle strength of quadriceps and hip flexors (measured via a handheld dynamometer) even following adjustment for age, BMI, vitamin D and PTH (26). Similarly in postmenopausal women, higher ucOC, but not tOC, was related to higher muscle mass (determined by bioelectrical impedance analysis) but only when adjusted for age and years since menopause (35). In a different study including postmenopausal osteoporotic women, a higher cOC, but not ucOC, was related to higher muscle mass (appendicular skeletal muscle mass divided by body mass index, ASM/BMI determined by DXA) (33). Furthermore, in that study a subgroup analysis including women with previous fractures, a higher cOC and ucOC was related to higher muscle mass and lower fall risk (33). Notably, the correlations performed in this study were not adjusted for confounding factors and this may limit the associations. Moreover, in adults with hypoparathyroidism treated with PTH, an increased ucOC/tOC ratio was associated with increased elbow extension force (unadjusted model) but no relationship was evident with 11 other muscle function variables (i.e. grip strength, timed up and go) (34). There was also no relationship with these variables and the absolute value of ucOC (34). Conversely, others reported that a higher ucOC was related to lower muscle mass in obese patients with chronic kidney disease (36). To our knowledge only one longitudinal study has been performed exploring this link between ucOC and muscle health. In that study, the authors reported that baseline ucOC and ucOC/tOC were not related to muscle mass at baseline or at 3-year follow up (37). There may be some explanations for such conflicting data. The women in one study had not fasted (26) and it is known that feeding can affect BTMs (31). Another study measured cOC and ucOC, but not tOC. Therefore, the authors were unable to express ucOC as a percentage of tOC, thereby limiting our understanding of these findings in general and the direct comparison of findings between these studies (33). The majority of studies are cross sectional in design, with findings being observational in nature. Therefore, there is no long term (greater than five years) longitudinal evidence to support previous study findings.

Moreover, other studies have reported that higher tOC is related to muscle health (501-504), yet similar to ucOC these findings are also conflicting. One reported that a higher tOC is related to higher muscle mass in middle-aged and older adults, but not when adjusted for age and BMI (502). Furthermore, in that study, in uni- and multivariate models, a higher tOC was an independent predictor of muscle mass, but only in a

subgroup of men who were hyperglycaemic. Others report that higher tOC is related to lower muscle mass and grip strength in middle-aged and older adults (503) but the correlations were unadjusted. In addition, two studies report that higher tOC was related to osteosarcopenia presence (a term used to describe combination low bone and muscle mass and muscle strength) (501, 504), with one of these studies reporting that higher tOC was associated with an increased likelihood of osteosarcopenia (504). ucOC was not measured in these studies.

Altogether, based on the observational data from these human studies, the relationships between ucOC with muscle mass and ucOC with muscle strength is unclear and whether this is related to long term risk for injurious falls is unknown. The same lack of knowledge applies to the tOC and cOC forms and the ucOC/tOC ratio. A variety of methods for the assessment of tOC and ucOC as well as different methods for the determination of muscle indices have been used, which may explain some of the conflicting findings. Lastly, these study findings may be dependent on the statistical analyses and models of adjustment used. For example, some used adjustments and others did not.

Table 2.6. Data from human trials: associations of ucOC with muscle mass, strength and function

Studies First author, (Year)	**Study population** Sample (n)	**Sample size (n)**	**Mean age** (yrs)	**Mean BMI** (kg/m^2)	**Assay**	**Main findings on muscle function**
Levinger, (26) *Cross sectional*	Postmenopausal women	**90**	75 yrs	27 kg/m^2	tOC and ucOC HAP method *not fasted	↑ **ucOC/tOC** associated with ↑ **muscle strength** (adj age BMI) ↔ ucOC, tOC
Lee, (35) *Cross sectional*	Postmenopausal women with and without metabolic syndrome (MetS)	**135** 52 MetS 83 Non-MetS	56 yrs, 55 yrs	26 kg/m^2 23 kg/m^2	tOC and ucOC: N-MID osteocalcin and human ucOC ELISA Kits *fasted	↑ **ucOC** associated with ↑ **muscle mass** only when adj. for age and menopause ↔ tOC
Drey, (501) *Cross sectional*	Osteosarcopenia, pre frail community dwelling older adults	**68** 19 Osteosarcopenia 14 Sarcopenia 17 Osteoporosis 18 Controls	78 yrs 76 yrs 81 yrs 81 yrs	Not reported	tOC immunoassay (Roche Diagnostics, Mannheim, Germany) *fasting, a.m	↑ **tOC** associated with **osteosarcopenia**
Harslof (34) *Randomised, placebo-controlled trial*	Patients with hypoparathyroidism who received PTH (100ug, daily) or placebo	**58** 30 Controls 28 PTH	51 yrs 54 yrs	28 kg/m^2 30 kg/m^2	ucOC- (ELISA) (Takara) *fasted	↑ **% change in ucOC/tOC** associated with ↑ **% change in elbow extension force** (unadjusted)
Gomes, (36) *Cross sectional*	Obese patients with chronic kidney disease	**39**	56 yrs,	31 kg/m^2	GLA and GLU OC ELISA (MK128 and MK118; Takara Bio Inc., Japan, respectively)	↑ **ucOC** associated with ↓ **muscle mass**

Studies First author, (Year)	**Study population** Sample (n)	**Sample size (n)**	**Mean age** (yrs)	**Mean BMI** (kg/m^2)	**Assay**	**Main findings on muscle function**
Shea, (37) *Cross sectional and Randomised, placebo controlled study*	Community dwelling older adults	**401** Female Vit K Female control Male Vit K Male control	 74 yrs 70 yrs 86 yrs 84 yrs	 29 kg/m^2 27 kg/m^2 28 kg/m^2 28 kg/m^2	tOC and ucOC- RIA-HAP method *fasted	Baseline ucOC, ucOC/tOC ↔ muscle mass (ALM, total lean mass) 3 year change in muscle mass (ALM, total lean mass) ↔ between groups
Xu, (502) *Cross sectional*	Middle-aged and older adults	**1742** 775 Male 967 Female	61 yrs	24 kg/m^2	tOC via ECLIA (Roche Diagnostics GmbH, Mannheim, Germany). *fasted	↑ **tOC** associated with ↑ **muscle mass** (SMI) (unadjusted) but ↔ when adj for age and BMI ↑ **tOC** associated with ↑ **muscle mass** only in men with hyperglycaemia not women, other groups based on obesity, dyslipidaemia ↑ **tOC** independent predictors of ↑ **muscle mass** in hyperglycaemic men (univariate & multivariate adj model)
Moriwaki, (503) *Cross sectional*	Community dwelling middle-aged and older adults	**253** 97 Male 157 Female	 75 yrs 74 yrs	 23 kg/m^2 22 kg/m^2	tOC: ECLIA (kit details not stated) *did not state if fasting or not	↑ **tOC** associated with ↓ **muscle mass** (SMI) and **grip strength** (unadj)

Studies First author, (Year)	**Study population** Sample (n)	**Sample size (n)**	**Mean age** (yrs)	**Mean BMI** (kg/m^2)	**Assay**	**Main findings on muscle function**
Fathi, (504) *Cross sectional*	Older adults	**397** 76 Osteosarcopenia 321 Non-osteosarcopenia	73 yrs 68 yrs	24 kg/m^2 29 kg/m^2	tOC ECLIA (Roche Diagnostics GmbH) *fasting	↑ **tOC** in **osteosarcopenia** vs non-osteosarcopenia ↑ **tOC** associated with increased likelihood of **osteosarcopenia**
Vitale, (33) *Cross sectional*	Non-diabetic, non-obese, postmenopausal osteoporotic women	**29**	72 yrs	23 kg/m^2	ucOC: EIA kit Gla-Type OC EIA kit, (Takara Bio Inc., Otsu-Shi, SHG, Japan) *fasting	↑ **cOC** associated with ↑ **ASM/BMI** ns ucOC In women who had fractured, a ↑ **cOC and ucOC** was related to ↑ **muscle mass** and **lower falls risk**

Key: tOC, total osteocalcin; ucOC, undercarboxylated osteocalcin; HAP, hydroxyapatite; BMI, body mass index; MetS, metabolic syndrome; ELISA, enzyme-linked immunosorbent assay; PTH, parathyroid hormone; Vit K, vitamin K; RIA, radioimmunoassay; ECLIA, electrochemiluminescence immunoassay; ALM, appendicular lean mass; SMI, skeletal muscle index

2.2.3 The link between CTX and P1NP and muscle function: effects of exercise

2.2.3.1 *The relationship between CTX and P1NP with muscle function*

To date, OC was the main focus in studies exploring bone and muscle interactions. Yet, other bone-derived factors including BTMs (i.e. CTX and P1NP) may be involved, but the evidence is conflicting. For instance, in older women CTX and P1NP were not linked to muscle strength (26). However, a potential limitation in this study was that the women did not fast overnight and it is known that feeding can affect BTMs (31). Other studies have shown that in older adults poorer mobility and balance are related to higher CTX and P1NP levels after adjustment for age and sex (47). Furthermore, serum P1NP but not CTX remained associated with poorer mobility after accounting for multiple confounders (i.e. vitamin D, PTH, renal function, nutrition), suggesting that P1NP may be more strongly related to immobility than CTX. A recent study in older adults with high risk of falls and fractures reported that a higher serum CTX was associated with poorer lower limb muscle function even after adjustment for multiple confounders (48). Altogether the data suggest that P1NP and CTX may be related to muscle function. As BTMs are already used clinically, they represent an easy to implement strategy to identify older adults with low muscle function. However, the direction of this relationship remains unclear and is probably influenced by numerous factors such as underlying bone pathophysiology (i.e. bone health status). In this thesis (***Study 4***) I explore the link between BTMs and muscle function in a representative, healthy community dwelling cohort of older adults who had not sustained fractures. In addition, the link between OC and muscle function, along with its relationship to the risk for injurious falls, is explored in **Study 2** in a large longitudinal cohort over a 15 year follow up.

2.2.3.2 *Essential characteristics for optimal loading of bone: an overview*

As previously discussed, bone and muscle mass are regulated predominantly by mechanical stimuli (505-507). Decreases in mechanical loading result in muscle atrophy. A similar response is seen in bone, where a shift in the balance in favour of bone resorption results in rapid bone loss (290-294, 508). As stated by Beck (509) "*age-related bone loss is a manifestation of the principle*". That is, most people as they age become inactive and progressively unload their bones, which predisposes them to osteoporosis. Older adults who maintain their young adult exercise levels tend to maintain their bone

mass (510, 511). These data suggest that the adaptive response to bone is proportional to mechanical strain (512, 513), with bone having the ability to change its mass, shape and properties in response to loads placed on it (514-517). For this reason exercise is a potent strategy to manage osteoporosis allowing the skeleton to withstand the loads and forces of everyday activities without sustaining fracture (285, 286, 518).

Bone strength is the outcome of many processes and factors involving the complex interplay between bone structure (trabecular, cortical bone), material properties (organic and inorganic) and structural properties (geometry), all determined by bone remodelling. Bone relies on muscle as one of its key osteogenic stimulants through such things as muscle contraction during exercise, which applies different forces to bone (***Figure 2.18***) (39). In addition, bone relies on mechanical strain derived from impact loading and gravity (258, 519-523).

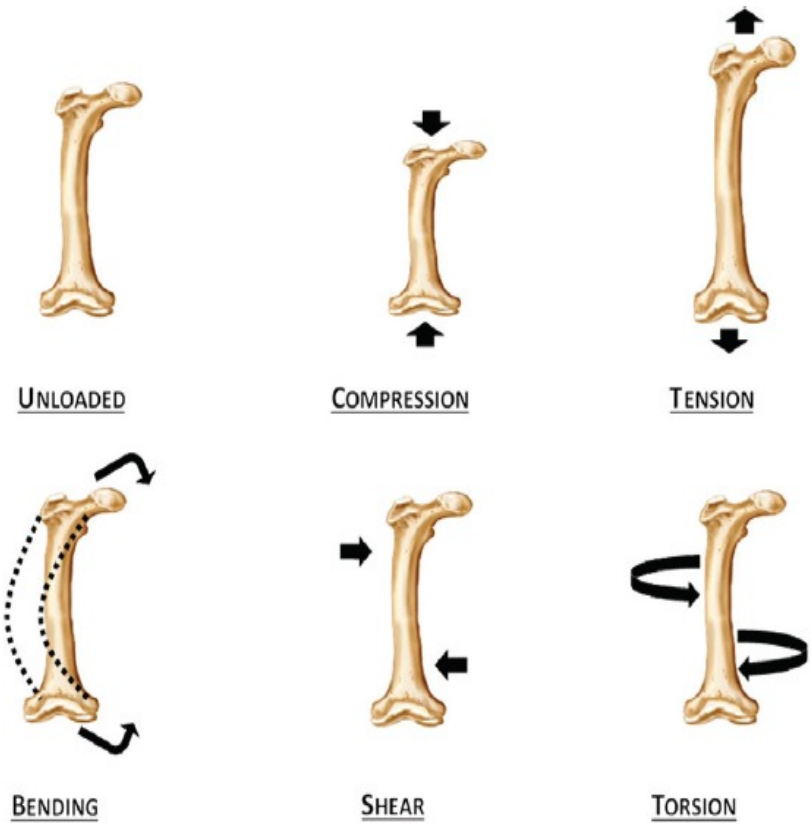

Figure 2.18 The different forms of mechanical strain applied to bone. Muscles contribute to the mechanical loading of bone via tensile force (from contracting muscles), compressive force (muscle contracting across joints) and bending force (experienced by long bones, i.e. with muscle generating force when lifting an object distal to the limb). Bone experiences deformations in response to loads (force) placed upon it, and bone cells sense strain, related to tissue deformation, fluid flow or other processes within the bone matrix. Strain describes how much deformation occurs in bone in response to force (i.e. compressive, tensile and torsional strain). Figure sourced from Hart et al.(258).

This action of muscle on the adaptive response of bone was first modelled by Wolff's law and Frost's mechanostat theories. According to those theories, mechanical loading is essential for bone strength (524-526) and consequently it was proposed that bone mass across the lifespan is dependent on skeletal muscle-derived mechanical loading (38, 39). Therefore, the key characteristics of a prescribed exercise program to optimise bone health were built upon evidence established from many animal studies. The evidence demonstrates that bone responds to loads that are: dynamic, not static (527), high in magnitude and applied rapidly (528, 529) in diverse and unusual patterns (530, 531), and that only few repetitions are required if the load is sufficient (531). Some evidence also suggests that loads interspersed with rest are more osteogenic than continuous loading, as a result of a desensitising effect on bone cells (515). The translation of these findings from animal studies has been confirmed by exercise studies in humans. For example, higher impact activities i.e. tennis, squash or badminton are more osteogenic than running or cycling (532). Altogether, the key loading features to optimise bone responses that form the principles of exercise prescription for osteoporosis prevention and management are depicted in (**Figure 2.19**) (258, 285, 509, 518). These characteristics were used to inform the choice for the exercise protocol used in **Study 4** to investigate bone-muscle interaction.

Figure 2.19 Optimal loading characteristics for bone responses forming the principles for exercise prescription.

2.2.3.3 *Why BTMs are used in response to exercise rather than BMD?*

BMD increases in response to the physical load, force and mechanical strain placed upon it. In an effort to address safety concerns during exercise, many clinical trials have used interventions of insufficient magnitude. As a result, the trials report conflicting results on bone strength (533-543). Based on the literature, only studies that have integrated multi-component programs show chronic, long-term effects on bone strength, as a result of the high intensity, progressive RT and impact weight bearing employed (534, 535, 544, 545). It can take many months, or even years, to see a change in BMD, which may explain some of the conflicting data (63). Some suggest that if BMD is maintained, a null response (not significantly negative or positive) could be considered a beneficial effect of the exercise response, but it does highlight that there is a need to examine more systematically the effect of exercise on bone remodelling. BMD measurement, via DXA, provides a static representation of bone strength and is thus a poor representation of the underlying metabolic dynamics (546). As such, investigating immediate effects of exercise and strain and load on bone and muscle interaction using BMD is not viable. In contrast to BMD, BTMs can respond to, and are released following, mechanical load and this response occurs very rapidly, even after an acute, single bout of exercise (section **2.2.4.3**).

2.2.3.4 *Exercise effects on BTMs*

A large body of research in younger, athletic populations demonstrates that exercise can alter BTMs (50, 51) but the benefits are less clear in older adults. A recent systematic review of osteoporotic cohorts reported that the beneficial effects of chronic, long-term exercise could in part be explained by altered BTMs (increasing bone formation and decreasing bone resorption markers) (52). Others have shown that BTMs are modified following exercise in older adults, even with null change in BMD in some cases. For instance, six months of PRT in older adults increased femoral neck BMD but only if performed at high intensity at 80% 1RM. However, bone turnover (tOC and BAP) increased following both high (80% of1RM) and low intensity PRT (50% of IRM), suggesting PRT alters BTMs which may over time lead to change in BMD (547). Additionally, 24 weeks of high intensity aquatic exercise in postmenopausal women increased BTMs (P1NP, CTX) but did not change BMD, compared to the control group

who exhibited increased CTX but decreased BMD, suggesting that exercise in this case attenuated BMD loss in a setting of increased bone remodelling (548). These studies support the concept that BTMs may be a better indicator of underlying bone metabolism than BMD *per se*.

While there is a large breadth of available data and review papers on BTM responses to acute exercise in younger populations (50, 51), there are limited studies in older adults (49, 53-56) and the findings are conflicting and unclear. In this population many studies performed only AE (49, 53, 55, 56) with very few performing RE (54). This is surprising given that the key characteristics for bone loading are more likely to be heightened with RE. Altogether, the evidence suggests that exercise can be used as a tool to examine bone-muscle crosstalk via BTMs. Nevertheless, there is a need to systematically review the evidence in older adult cohorts to understand if responses are different based on exercise mode, intensity, age and sex. This is presented in ***Study 3, chapter 6*** of this thesis. In addition, a randomised crossover trial is presented as a part of this body of research to uncover if exercise mode has differing effects on BTMs and to investigate the bone-muscle interaction using ucOC and BTMs (***Study 4, Chapter 6***).

2.2.3.5 *The link between muscle function and exercise responses of BTMs*

While the evidence demonstrates BTMs can be modified by exercise (long term and after a single session) (50, 423), the link between BTMs and muscle strength and function remains unclear and conflicting. Given the close interaction described between the two organs (38-40) it is possible that underlying muscle physiology (high or low muscle mass or function) may be linked to BTM levels and partially explain BTM responses to altered mechanical load through exercise (40-42). To our knowledge, this had not been explored prior to **Study 4** of this thesis.

2.3 Gaps in the literature

Based on this literature review this thesis will address the following gaps:

- To date, there are no normative age-reference ranges for the OC forms: cOC and ucOC. There is also not reference range for the OC ratios: ucOC/tOC and cOC/tOC. It's also unknown what the age effects are on these OC forms and ratios. This forms the aims of **Study 1, Chapter 3**.

- There are no longitudinal data to support current evidence of whether there is a relationship between ucOC and muscle health and whether this is related to hazardous outcomes such as the risk of injurious falls. This forms the aims of **Study 2, Chapter 4.**
- It is unknown how BTMs are influenced by acute exercise in middle-aged and older adults, and whether these responses are specific to exercise mode, intensity, age or sex. This forms the aims of **Study 3, Chapter 5.**
- There is a limited number of acute exercise studies on BTM responses in older adults, with an even smaller number based on RE protocols. Additionally, it is unknown whether BTM responses can be influenced by the baseline status of muscle function (i.e. lower or higher muscle mass or strength). This forms the aims of **Study 4, Chapter 6.**

2.4 Aims and hypotheses

The overall aim of this thesis is to define the general ageing effect on ucOC and to uncover whether its relationship with muscle function and hazardous outcomes such as falls risk is limited to ucOC, or related to BTMs in general in older adults. This is investigated in four studies. The specific hypotheses and aims of each study are listed below.

Study 1 (chapter 3)

Aim 1: To determine how tOC, ucOC, cOC and the ratios ucOC/tOC and cOC/tOC change with age in adult men and to define normative ranges of the OC forms and OC ratios in this population. The hypothesis is that ucOC would follow a negative linear relationship with age.

Study 2 (chapter 4)

Aim 1: To perform a cross-sectional study in a large cohort of older women to determine the relationship between ucOC and the ucOC/tOC ratio with muscle parameters.

Aim 2: To perform longitudinal analysis in a large cohort of older women to determine a) whether baseline ucOC and ucOC/tOC ratio is related to the long-term change in muscle parameters and b) whether baseline ucOC or ucOC/tOC is related to risk for injurious falls over 14.5 years. We tested the hypothesis that in a large cohort of older

women a higher ratio of ucOC/tOC would be associated with reduced muscle function (strength and physical function) and an increased risk of long-term risk of falls-related hospitalisations.

Study 3 (chapter 5)

Aim 1: to examine through systematic review the effects of acute exercise on BTMs in adults over 50 years of age and to determine if middle-aged and older adults respond differently**.**

Aim 2: to understand whether these effects were specific to exercise modality, exercise intensity, sex or BTM. We tested the hypothesis that older and middle-aged adults would have different BTM responses to acute exercise and these responses would be specific to exercise modality, intensity, sex or BTM.

Study 4 (chapter 6)

Aim 1: to perform cross-sectional analyses to determine the relationship of baseline determinants of muscle mass and function with tOC, ucOC, CTX and P1NP.

Aim 2: to determine the effect of acute aerobic (AE) and resistance (RE) exercise on bone biomarker responses.

Secondary aim 1: to explore if baseline muscle mass and function are related to the bone biomarker responses following acute exercise.

Secondary aim 2: to explore if bone biomarker responses following acute exercise are related to the glucose-lowering effect of exercise. Our hypothesis was that higher muscle function would be related to higher BTMs at baseline, and that those with higher muscle function would have lower BTM responses to acute exercise. We also hypothesised that RE would elicit higher BTM responses compared to AE. We hypothesised that following exercise the change in ucOC, but not in other BTMs would be related to lower levels of post-exercise glucose.

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Chapter 3: Osteocalcin and its forms across the lifespan in adult men

Context

To date, it is unclear what is the ageing effect on tOC some report it increases, others decreases with age, and, it is unknown what is the ageing effect on OC forms, with no normative, age-referent ranges available. The purpose of this study was to determine the ageing effect on tOC, ucOC, ucOC/tOC ratio across the lifespan.

The following paper has been published:

Smith, C., Voisin, S., Al Saedi, A., Phu, S., Brennan-Speranza, T., Parker, L., Eynon, N., Hiam, D., Yan, X., Scott, D., Blekkenhorst, L. C., Lewis, J. R., Seeman, E., Byrnes, E., Flicker, L., Duque, G., Yeap, B. B., & Levinger, I. (2020). Osteocalcin and its forms across the lifespan in adult men. *Bone*, *130*, 115085. https://doi.org/10.1016/j.bone.2019.115085

Please see **Appendix 2** for the published version of this manuscript.

It was also presented at the following conferences:

- Australian Institute for Musculoskeletal Sciences (AIMSS) conference (2019), oral presentation*
- Victoria University, Higher Degree by Research conference, oral presentation

I received two awards for this study, receiving best oral presentation at the AIMSS conference, and best oral presentation at the VU HDR conference

OFFICE FOR RESEARCH TRAINING, QUALITY AND INTEGRITY

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

1. PUBLICATION DETAILS (to be completed by the candidate)

Title of Paper/Journal/Book: Osteocalcin and its forms across the lifespan in adult men

Surname: Smith First name: Ca

Institute: Institute for Health and Sport Candidate's Co

Status:

Accepted and in press: ☐ Date:

Published: ☑ Date: 14/1

2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be inclu ed in the HDR Policy and related Procedures – policy.vu.edu.au.

Cassandra Smith Digitally signed by Cassandra Smith Date: 2022.03.19 11:24:21 +11'00' | 19/03/2022

Signature **Date**

3. CO-AUTHOR(S) DECLARATION

In the case of the above publication, the following authors contributed to the w

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in t or interpretation of at least that part of the publication in their field of e
2. They take public responsibility for their part of the publication, excep nor who accepts overall responsibility for the publication;

3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to a) granting bodies, b) the editor or publisher of journals or other publications, and c) the head of the responsible academic unit; and
5. The original data will be held for at least five years from the date indicated below and is stored at the following **location**(**s**):

Victoria University

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Bu B. Yeap	2.5	Data collection, editing of manuscript		25.3.22
Itamar Levinger	10	Overall study design, editing o manuscript		21/03/2022

Osteocalcin and its forms across the lifespan in adult men

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3.1 Abstract

Purpose: Osteocalcin (OC), an osteoblast-specific secreted protein expressed by mature osteoblasts, is used in clinical practice and in research as a marker of bone turnover. The carboxylated (cOC) and undercarboxylated (ucOC) forms may have a different biological function but age-specific reference ranges for these components are not established. Given the different physiological roles, development of reference ranges may help to identify people at risk for bone disease.

Methods: Blood was collected in the morning after an overnight fast from 236 adult men (18 to 92 years old) free of diabetes, antiresorptive, warfarin or glucocorticoid use. Serum was analyzed for total osteocalcin (tOC) and the ucOC fraction using the hydroxyapatite binding method. cOC, ucOC/tOC and cOC/tOC ratios were calculated. Reference intervals were established by polynomial quantile regression analysis.

Results: The normal ranges for young men (≤ 30 years) were: tOC 17.9-56.8 ng/mL, ucOC 7.1-22.0 ng/mL, cOC 8.51-40.3 ng/mL (2.5^{th} to 97.5^{th} quantiles). Aging was associated with a "U" shaped pattern for tOC, cOC and ucOC levels. ucOC/tOC ratio was higher, while cOC/tOC ratio was lower in men of advanced age. Age explained ~31%, while body mass index explained ~4%, of the variance in the ratios.

Conclusions: We have defined normal reference ranges for the OC forms in Australian men and demonstrated that the OC ratios may be better measures, than the absolute values, to identify the age-related changes on OC in men. These ratios may be incorporated into future research and clinical trials, and their associations with prediction of events, such as fracture or diabetes risk, should be determined.

Key words: Osteocalcin, aging, reference ranges, bone, bone turnover

3.2 Introduction

Osteoporosis affects 1.2 million Australians, with a further 6.3 million affected by osteopenia, with both rates projected to rise in the years to come (1, 2). Six percent of men aged over 50 years have osteoporosis, increasing to 13% in those over 70 years (1, 2). Circulating levels of bone turnover markers (BTMs) are used in research and clinical practice to predict fracture risk (3-5). Reference intervals and treatment targets of BTMs for older women, based on premenopausal data, have been extensively characterized, however, only a few studies are available for men (6-8).

Serum total osteocalcin (tOC), an osteoblast-specific secreted protein expressed by mature osteoblasts, is the most abundant, non-collagenous protein found within the bone matrix and is used as a BTM (9, 10). tOC exists in the circulation in two major forms; γ-carboxylated (cOC) and undercarboxylated (ucOC) which lacks γ-carboxylation at one or more sites (11). cOC is thought to be predominately located in bone, whereas ucOC may participate in glucose metabolism, influencing muscle mass and strength (12-22). Previous studies indicate circulating tOC is highest in early adulthood, lower in mid-life, and with mixed results shown in older adults (23-31). Despite the differences in the biological functions of the OC forms, few studies report both forms and tOC levels, or their age-specific distributions. Consequently, normal ranges of OC forms and ratios (ucOC, cOC, ucOC/tOC, cOC/tOC) in men are not quantified.

The aims of the current study were to determine how tOC, ucOC, cOC and the ratios ucOC/tOC and cOC/tOC change with age in adult men and to define normative ranges of the OC forms and OC ratios in this population.

3.3 Material and Methods

3.3.1 Study Population

This is a cross-sectional study representative of men across the adult lifespan, utilizing collected fasted baseline (resting) sera samples of a total of 236 men aged 18 to 92 years. The datasets include data from the following studies: (a) the Health In Men Study (HIMS), a population-based cohort study, comprising of 4,248 men aged 70 to 89 years, assessed in 2001-04, who have been followed-up since initial recruitment in 1996 (Perth, Western Australia); From men in HIMS who had previously had tOC and ucOC assessed (549), after excluding men with diabetes, heart disease or osteoporosis, 99 men

were randomly selected for the current study; (b) the Nepean Osteoporosis and Frailty (NOF) study, a cross-sectional study of older adults with frailty and other comorbidities (Western Sydney, Australia). A total of 23/76 samples were eligible after exclusions for diseases (i.e. diabetes) and medications known to affect OC and bone metabolism, 23 were eligible (550); (c) exercise studies at Victoria University investigating bone health, comprising 20 healthy men aged 21 to 70 years (30, 551); (d) the Gene Smart Study, is an ongoing international, multi-center study that is a part of the recently established ATHLOME Consortium (552, 553). To date (April 2019), 94 men have completed the study. At the time of establishing the current study only 74 samples from healthy, young men (aged 18 to 45 years) were collected and were included in the current study; and (e) the Vegetable Intake and Blood Pressure (VIABP) study is a randomised, controlled, cross-over study of 30 non-smoking, non-diabetic participants (20 men, 10 women) with pre-hypertension or untreated grade 1 hypertension, only samples from men, aged 40 to 74 years (first baseline visit), were included (554). All volunteers signed a consent form for participation in the respective studies.

In total, of the 236 men included in this study, 17 men from the NOF study and 5 men from the VIABP used medications including antihypertensives (NOF, n=9); antiplatelets (NOF, n=2), nitrates (NOF, n=2); statins (NOF, n=7; VIABP, n=2); ventolin (NOF, n=2); proton pump inhibitors (NOF, n=6; VIABP, n=1); diuretics (NOF, n=2); non-steroidal anti-inflammatory drugs (NSAIDs) (NOF, n=2; VIABP, n=2); opioids (NOF, n=1); anticholinergic/anti-muscarinic (NOF, n=1) and vitamin D/calcium (NOF, n=2).

3.3.2 Quantification of serum osteocalcin (tOC) and undercarboxylated osteocalcin (ucOC)

The stored sera samples were selected on the following criteria: a) samples collected in the morning following an overnight fast (to minimize circadian variation); b) samples were analyzed at the same laboratory, by the same technician and following the same methodology; and c) all samples were collected as serum and kept in aliquots in long term storage at -80C until assayed and no freeze-thaw cycles previously reported.

Frozen serum samples from each clinical trial were obtained from long-term storage and analyzed using identical technique and equipment, and performed by the same technician. Serum tOC was measured using an automated immunoassay (Elecsys 170;

Roche Diagnostics). Serum ucOC was measured by the same immunoassay after absorption of carboxylated OC on 5mg/mL hydroxyl-apatite slurry, following the method described by Gundberg et al. (11) and Chubb et al. (8).

The Elecsys N-MID Osteocalcin assay uses two monoclonal antibodies specifically directed against epitopes on the N-MID fragment and the N-terminal fragment. The assay hence detects the stable N-MID fragment as well as the (still) intact OC. The test is non-dependent on the unstable C-terminal fragment (amino acids 43 – 49) of the OC molecule and thus ensures constant measurement results under routine conditions in the laboratory. Test Principle (from Roche N-MID Osteocalcin product insert). Sandwich immunoassay – assay duration 18 minutes. 1st incubation: 20uL of sample, a biotinylated monoclonal N-MID OC specific antibody and a monoclonal N-MID OC -specific antibody labelled with a ruthenium complex [Tris (2,2'-bipyridyl)ruthenium(II)-complex; $Ru(bpy)_3^{2+}$] react to form a sandwich complex. 2nd incubation: after addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell M. Application of a voltage to the electrode then induces a chemiluminescent emission which is measured by a photomultiplier. Results are determined via a calibration curve which is generated by 2-point calibration and a master curve provided via the reagent barcode. The reagents used for the measurement of OC were the Roche N-mid Osteocalcin (Roche Diagnostics, Mannheim) on the Roche E170 Analyzer (Elecys 170; Roche Diagnostics). This is the same reagent and instrument used in previous studies by Chubb et al (8) and Levinger et al (15, 18, 38). The hydroxyapatite used was Calbiochem Hydroxyapatite, Fast Flow catalog # 391947 as described by Gundberg et al (11) and as used in our previous work (15, 18, 38).

Using commercial control material (Roche Precivaria controls level 1 and 2), the following inter-assay variability was seen over 16 analytical runs for OC on the Roche E170: N = 16, mean 19.02, SD 0.33, CV 1.71%; N = 16, mean 91.41, SD 3.01, CV 3.29%. Using an OC standard material purchased from Sigma chemicals spiked into OC free serum, the following interassay variability was seen over 7 analytical runs for the tOC and % binding to hydroxyapatite: High concentration N = 7, mean total 193.43, SD 14.70, CV 7.60%; High concentration N = 7, mean % bound 79.17%, SD 2.21, CV 2.80%; Low concentration N = 6, mean total 18.21, SD 1.78, CV 9.78%; Low concentration N = 6,

mean % bound 73.94%, SD 4.62, CV 6.25%. This % binding was similar to that seen in previous studies (39-41).

3.3.3 Statistical Analysis

All statistical analyses were performed using R version 1.1.453 (42). We initially intended to use quantile regression as previously published (43) to generate 95% reference ranges for the bone markers and ratios within the cohort, but we noted that age strongly modified the reference ranges. Therefore, instead of using arbitrary age cut-offs and splitting our cohort into smaller age groups, we performed polynomial regression of degree 2 for tOC, cOC and ucOC, and simple regression for the ratios cOC/tOC and ucOC/tOC. We generated 95% reference ranges for each of the bone markers as a continuous function of age, with the predict() function. The 95% reference ranges are easily readable as red dashed lines on the individual figures. We also added body mass index (BMI) as a covariate to each of the models to determine whether adjustment for BMI was required. Reference ranges for men <30 years were given as the 2.5^{th}-97.5^{th} quantiles of each bone marker. We also report the 95% CI for the upper and lower limits of the reference ranges. All data are presented as mean ± SD. For all statistical analyses, p values <0.05 were considered statistically significant.

3.4 Results

A total of 236 men were included with a mean age of 58.1 ± 21.7 years and BMI of 26.2 ± 3.8 kg/m^2 (**Table 3.1**, sample of men per decade of age are presented in **Table 3.2**). tOC, ucOC and cOC (**Figure 3.1A-C**) all displayed a "U shaped" relationship across the aging continuum, with lowest levels observed around 55 years of age. Specifically, from 18 to 59 years old, tOC levels diminished in a non-linear fashion from ~42 ng/mL to ~18 ng/mL; tOC levels were higher after 59 years of age in a non-linear fashion, attaining ~24.2 ng/mL at 80 years old. ucOC and cOC levels show similar associations with age. From 18 to 52 years old, ucOC levels diminished from ~14.7 ng/mL to ~8.7 ng/mL; ucOC levels increased after 52 years of age, attaining ~12.6 ng/mL at 80 years old. From 18 to 63 years old, cOC levels diminished from ~27.1 ng/mL to ~8.3 ng/mL; cOC levels were higher after 63 years of age, attaining ~11.6 ng/mL at 80 years old.

Table 3.1 Descriptive characteristics of our cohort of fasted adult men

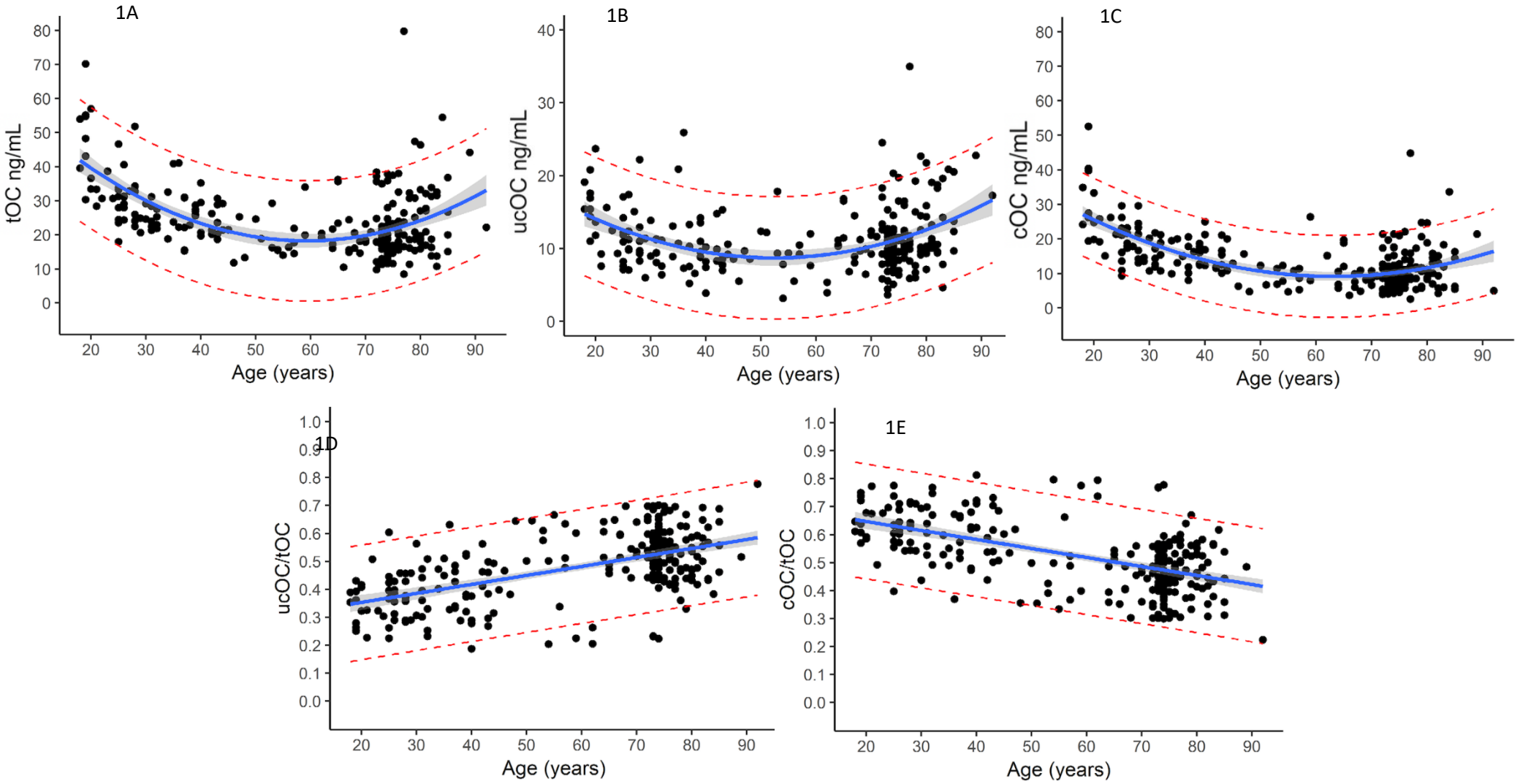
	Entire cohort (mean ± SD)
Sample (n)	236
Age (years)	58.14 ± 21.73
BMI (kg/m^2)	26.18 ± 3.83
tOC (ng/mL)	24.78 ± 10.58
cOC (ng/mL)	13.51 ± 7.69
ucOC (ng/mL)	11.26 ± 4.48
ucOC/tOC	0.48 ± 0.12
cOC/tOC	0.52 ± 0.12

All data reported as mean ± SD. BMI, Body mass index; tOC, total osteocalcin; ucOC, undercarboxylated osteocalcin; and cOC; carboxylated osteocalcin.

Table 3.2 Total number of men per decade of age

Age group	Total n
< 20 years	8
20 to 29 years	32
30 to 39 years	28
40 to 49 years	18
50 to 59 years	13
60 to 69 years	16
70 to 79 years	93
80 to 89 years	27
> 90 years	1

Figure 3.1 Relationship between age and circulating levels of tOC, ucOC and cOC, and the OC-ratios with confidence and prediction intervals in healthy adult men



Mean and SD for the absolute values of OC forms were calculated to determine the dispersion of individual values between young (< 30 years) and older adults (> 70 years); tOC in young men was lower than older adults (35.31 ±11.56 versus 22.33 ± 10.17); ucOC was similar in young and older men (12.66 ± 4.29 versus 11.59 ± 4.79) and cOC was higher in younger men compared to older (22.65 ± 8.69 versus 10.74 ± 6.22).

In contrast to the individual forms, there was an incremental rise in ucOC/tOC ratio across age (**Figure 3.1D**) while the cOC/tOC ratio was lower (**Figure 3.1E**). Adjusting for the effect of BMI, on average, men have 0.3 ± 0.03% lower cOC/tOC ratio per decade; conversely, increments of 0.3 ± 0.03% of ucOC/tOC per decade of age were observed. Age explained ~31% of the variance while BMI explained ~4% of the variance in the ratios. BMI was not associated with individual measures of tOC, cOC and ucOC and as such was not adjusted for in those models (**Table 3.3**).

Table 3.3 β estimates: Regression coefficients for all OC forms and ratios in adult men

	β estimate Regression coeff. Estimate ± Std. error	**p value**	**Adjusted R^2**
tOC (ng/mL)			
Age	-1.6 ± 0.20	**$4.20 \times 10^{-14***}$**	**0.29**
Age^2	0.014 ± 0.0019	**$7.73 \times 10^{-12***}$**	
cOC (ng/mL)			
Age	-1.11 ± 0.138	**$4.30 \times 10^{-14***}$**	**0.39**
Age^2	0.00877 ± 0.00130	**$1.11 \times 10^{-10***}$**	
ucOC (ng/mL)			
Age	-0.53 ± 0.097	**$9.98 \times 10^{-8***}$**	**0.11**
Age^2	0.0051 ± 0.0091	**$7.25 \times 10^{-8***}$**	
ucOC/tOC			
BMI	0.00692 ± 0.00173	**$8.49 \times 10^{-5***}$**	**0.35 (adjusted to BMI)**
Age	0.00300 ± 0.000305	**$< 2 \times 10^{-16***}$**	**0.31 (unadjusted)**
cOC/tOC			
BMI	-0.00692 ± 0.00173	**$8.49 \times 10^{-5***}$**	**0.35 (adjusted to BMI)**
Age	-0.00300 ± 0.000305	**$< 2 \times 10^{-16***}$**	**0.31 (unadjusted)**

*** p-value ≤0.01, * p-value ≤0.05. BMI, Body mass index; tOC, total osteocalcin; ucOC, undercarboxylated osteocalcin; and cOC; carboxylated osteocalcin

The recommended reference ranges (2.5th to 97.5th quantiles) and 95% confidence limits for the lower and upper limits, based on the data of the young (<30 years old), healthy men are presented in **Table 3.4** for all OC forms and ratios.

Table 3.4 Normal reference ranges and 95% confidence limits for a reference cohort of young, healthy men

	Reference range (2.5th-97.5th quantiles)	95% CI for the limits of the reference range	
		Lower limit	Upper limit
tOC (ng/mL)	17.85 - 56.78	14.30 – 20.65	54.00 - 70.20
ucOC (ng/mL)	7.07 - 22.03	6.00 - 7.58	17.60 - 23.68
cOC (ng/mL)	8.51 - 40.33	4.28 – 10.75	33.33 - 52.60
ucOC/tOC	0.23 - 0.60	0.22 - 0.26	0.54 - 0.70
cOC/tOC	0.39 - 0.77	0.29 - 0.44	0.74 - 0.78

tOC, total osteocalcin; ucOC, undercarboxylated osteocalcin; and cOC; carboxylated osteocalcin

3.5 Discussion

We report that in adult men (a) for all OC forms, aging was associated with a u-shape pattern expressed across the lifespan and, (b) age accounted for ~30% of the inter-individual variability in the ucOC/tOC and cOC/tOC ratios.

Circulating tOC is used in clinical practice as one of the measures to assess bone disease and as a surrogate measure for bone turnover (9, 10, 44, 45). As reported (23, 25, 27), we confirm that circulating tOC follows a u-shape pattern across the lifespan with high values in young individuals and in older individuals. This pattern with aging was also observed in the current study for cOC and ucOC. Clinically, this observed pattern limits the capacity of using the absolute concentration of OC forms for risk stratification. For instance, mean circulating tOC concentrations of 30 ng/mL can be observed in a 30 year old and 70 year old man, but in younger men it may indicate modelling and remodelling associated with the consolidation of bone, while in older men it may indicate increased bone remodelling, bone loss and emerging bone fragility (4, 9-13, 16-22, 46).

We observed that ucOC was higher in older adults compared to middle aged adults (fig 1), but similar in young men. The higher levels of ucOC in older adults is intriguing, ucOC has been reported to be involved in both glucose and lipid metabolism, and low

ucOC is associated with an increased risk for cardiovascular disease and diabetes (12, 13, 21, 22, 47, 48), even after adjustment for body mass index (13). As such, one may speculate that older adults will have lower ucOC, compared to middle aged individuals, as age is associated with increased risk for diabetes, this however was not supported by the evidence. Whether the increase in ucOC in older adults is due to a reduction in vitamin K intake or a potential compensatory mechanism to maintain normal circulating glucose levels in older adults is not clear and should be explored in future studies.

In contrast to absolute OC values, OC ratios (cOC/tOC and ucOC/tOC) may be more sensitive to the physiological changes associated with aging. In the current study cOC/tOC ratio was significantly lower in advanced age. Others have demonstrated that lower cOC/tOC ratio can predict fracture risk particularly in older men (49). Taken together, our data indicates that the ratio may be more useful for risk stratification and likely provides a better reflection of disease status including osteoporosis, fracture risk or metabolic diseases. Future research that includes clinical outcomes is required to confirm this.

The reference ranges for tOC have previously been established and are commonly used in clinical practice (25, 50). In the current study, we used data from young, healthy men to estimate the reference range for "optimal" levels as it corresponds to the time where bone mass peaks (51). Our reported reference range for young, healthy men for tOC was 17.9 to 56.8 ng/mL (2.5^{th} to 97.5^{th} quantiles), which is slightly lower than the clinical standard. We have used fasting and morning sampling times to minimize the effect of diurnal variation and feeding, both of which are reported to effect BTMs (52-54). This may suggest the references range used in clinical practice appears to be acceptable, however may need to be slightly adjusted.

As described above, assessing tOC limits the capacity to differentiate men based on age and to interpret underlying pathophysiology, therefore better clinical differentiation of this protein and its potential biological effects are needed (22-28, 55). We are not aware of any published reference interval data across the adult lifespan in men for cOC, ucOC or the ratios of cOC/tOC and ucOC/tOC. One study published data distributions for cOC and cOC/tOC (49), and another published reference intervals for ucOC and ucOC/tOC (8), however both studies were performed in older men >70 years old. We propose that the ratios of ucOC/tOC and cOC/tOC should be used in both clinical practice, and in future research, as they are potentially more robust measures to a)

distinguish the effect of aging on OC forms, and b) better understand the underlying aberrant physiology and biological action of OC in general.

3.5.1 Limitations

There are some potential limitations of our study. The current study focused on ranges of OC levels in men across the adult lifespan aged 18 to 92 years by combining separate study cohorts with different protocols for inclusion and exclusion criteria's and with different geographical locations which may introduce bias in our results. Whilst this study encompasses a large age range of adult men, there is only a small number of men in the youngest and oldest groups. However, the samples used in the current study were all from men without diseases (i.e. diabetes and osteoporosis) and medications (i.e. bisphosphonates and glucocorticoids) known to effect OC and bone metabolism. We have used the methods proposed by Gundberg et al. (11) to analyze *c*arboxylated and uncarboxylated OC, which is considered as the gold standard. However, different research groups use different methodologies to analyze ucOC and the levels depend on the technical details including antibody, specific surface of the hydroxyapatite, amount of the apatite or ELISA used. As such, the reference ranges calculated in the current study may not reflect the levels analyzed with different techniques. The study is also cross-sectional in nature and does not include clinical measures hence, we cannot ascertain whether these reference ranges are associated with incident or prevalence of disease. In the current study the ucOC/tOC in young men was relatively high. It is not clear why the fraction of ucOC was high and plausible explanations may include a non-specific binding to C-terminal fragments which do not contain GLA or diet with low vitamin K, which is required for OC carboxylation. Lastly, several factors may affect circulating OC levels including vitamin D or K, and although we did include people on vitamin K supplementation, we did not measure it.

3.5.2 Strengths

The strengths of our study include a full adult age range in men, a population at higher risk of cardiovascular diseases and poorer outcomes after bone fracture (56-58). Additionally, all samples were collected at the same time of day and in a fasted state. Therefor this is the first step in the validation of reference ranges for all OC forms and

ratios in adult men. In addition, all OC analysis was completed by the same technician, in the same laboratory and utilizing the same methodology and assays, minimizing variation due to technical error. The methods used in this study are automated and widely available however, the reference ranges proposed are only valid for the measurement of OC using the same assay, technical aspects and methodology. Measurement of OC may also be different according to countries and, therefore may limit the generalizability of the data. Future studies should explore the effect of aging on OC forms in women as the ranges and pattern of change across the adult lifespan may be different than observed for men.

3.5.3 Conclusion

In summary, we have defined normal reference ranges for the OC forms and demonstrated that the OC ratios may be better measures, than the absolute values, to identify the aging effect on OC in men. These ratios may be incorporated into future research and clinical trials, and their associations with prediction of events, such as fracture or diabetes risk, should be determined.

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Chapter 4: Higher undercarboxylated to total osteocalcin ratio is associated with reduced physical function and increased 15-year falls-related hospitalizations: the Perth longitudinal study of aging women

Context:

The manuscript in study 1 identified that total circulating levels of OC, including cOC and ucOC follow a u shape pattern with aging, while the ratio ucOC/tOC increases with age in adult men. Combined with the available evidence that demonstrated a potential relationship between ucOC and muscle function, we sought to understand in this chapter whether higher ucOC/tOC ratio would be related to poorer physical performance and injurious-falls in a large 15 year follow up study in older women. This study provided the first longitudinal evidence of a relationship between ucOC, physical function and falls risk.

The following manuscript has been published. Please see **Appendix 3** for the published version of this manuscript.

Smith, C., Lewis, J. R., Sim, M., Lim, W. H., Lim, E. M., Blekkenhorst, L. C., Brennan-Speranza, T. C., Adams, L., Byrnes, E., Duque, G., Levinger, I., & Prince, R. L. (2021). Higher Undercarboxylated to Total Osteocalcin Ratio Is Associated With Reduced Physical Function and Increased 15-Year Falls-Related Hospitalizations: The Perth Longitudinal Study of Aging Women. *Journal of bone and mineral research*, *36*(3), 523–530. https://doi.org/10.1002/jbmr.4208

It was also selected and presented at the following conferences

- *American Society for Bone and Mineral Research conference (2020)**
- *International Osteoporosis Foundation*
- *Australian and New Zealand Bone and Mineral Society conference (2021)*

*I also received a prestigious Young Investigator award from the *American Society for Bone and Mineral Research* for this work.

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DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

1. PUBLICATION DETAILS (to be completed by the candidate)

Title of Paper/Journal/Book:	Higher Undercarboxylated to Total Osteocalcin Ratio Is Associated With Reduced Physical Functio Increased 15-Year Falls-Related Hospitalizati The Perth Longitudinal Study of Aging Wome
Surname:	Smith
First name:	Ca
Institute:	Institute for Health and Sport
Candidate's Co	

Status:

Accepted and in press:	☐	Date:
Published:	☑	Date: 03/1

2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be inclu ed in the HDR Policy and related Procedures – policy.vu.edu.au.

Signature	Date
Cassandra Smith Digitally signed by Cassandra Smith Date: 2022.03.19 11:24:21 +11'00'	19/03/2022

3. CO-AUTHOR(S) DECLARATION

In the case of the above publication, the following authors contributed to the w

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in t or interpretation of at least that part of the publication in their field of e
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3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to a) granting bodies, b) the editor or publisher of journals or other publications, and c) the head of the responsible academic unit; and
5. The original data will be held for at least five years from the date indicated below and is stored at the following **location**(**s**):

Victoria University and Edith Cowan University

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Name(s) of Co-Author(s)	Contribution (%)	Nature of Contribution	Signature	Date
Itamar Levinger	10	Analysis/interpretation, editing of manuscript	[redacted]	21/03/2022

Updated: September 2019

This is the peer reviewed version of the following article: Smith, C., Lewis, J.R., Sim, M., Lim, W.H., Lim, E.M., Blekkenhorst, L.C., Brennan-Speranza, T.C., Adams, L., Byrnes, E., Duque, G., Levinger, I. and Prince, R.L. (2021), Higher Undercarboxylated to Total Osteocalcin Ratio Is Associated With Reduced Physical Function and Increased 15-Year Falls-Related Hospitalizations: The Perth Longitudinal Study of Aging Women. J Bone Miner Res, 36: 523-530., which has been published in final form at https://doi.org/10.1002/jbmr.4208 . This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions. This article may not be enhanced, enriched or otherwise transformed into a derivative work, without express permission from Wiley or by statutory rights under applicable legislation. Copyright notices must not be removed, obscured or modified. The article must be linked to Wiley's version of record on Wiley Online Library and any embedding, framing or otherwise making available the article or pages thereof by third parties from platforms, services and websites other than Wiley Online Library must be prohibited.

Higher undercarboxylated to total osteocalcin ratio is associated with reduced physical function and increased 15-year falls-related hospitalizations: The Perth Longitudinal Study of Ageing Women

Running title: Osteocalcin, muscle function and falls

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4.1 Abstract

Evidence from animal models suggests that undercarboxylated osteocalcin (ucOC) is involved in muscle mass maintenance and strength. In humans, the ucOC to total (t)OC ratio, may be related to muscle strength and, perhaps physical function and falls risk, but data are limited. We tested the hypothesis that ucOC and ucOC/tOC ratio are associated with muscle function (muscle strength and physical function) in older women and 15-year falls-related hospitalizations. Serum tOC and ucOC were assessed in 1261 older women (mean age 75.2 ± 2.7 years) forming the Perth Longitudinal Study of Ageing Women (1998 to 2013). Timed-up-and-go (TUG) and grip strength were assessed at baseline and at 5-years. Falls-related hospitalizations (14.5-year follow-up) was captured by the Hospital Morbidity Data Collection, via the Western Australian Data Linkage System. At baseline, women with higher ucOC/tOC ratio (Quartile 4) had slower TUG performance compared to Quartile 1 (~0.68 secs, $p<0.01$). Grip strength and 5-year change of TUG and grip were not different ($p>0.05$) between quartiles. Fear of falling limiting house, outdoor, and combined activities was significantly different across quartiles ($p<0.05$). Higher ucOC/tOC was significantly associated with poorer TUG performance at baseline and 5-year change in performance, increased walking aid use, and fear for falling (all $p<0.05$). Higher ucOC was related to lower grip strength at baseline ($p<0.05$), but not 5-year change in strength. Those with the highest ucOC/tOC had greater falls-related hospitalizations (unadjusted log rank $p=0.004$) remaining significant after adjusting for key variables (HR 1.31, 95% CI 1.09-1.57, $p=0.004$). We identified a large proportion of older women with high ucOC/tOC ratio that had reduced physical function, including its long-term decline and increased risk of falls-related hospitalizations. Early identification of women at higher risk can enable prevention and intervention strategies to occur, reducing risk for injurious falls.

Key words: aging; skeletal muscle; bone-muscle interactions; sarcopenia

4.2 Introduction

After the 4th decade of life, there is a rapid loss of muscle function before declines in muscle mass are evident [1-3]. Muscle function is used to describe the combination of muscle strength and physical function. Poor muscle function is associated with increased falls and fractures risk, functional disability, loss of independence, and early mortality [4-7]. Excess morbidity and mortality arising from the increased burden of compromised muscle function in community-dwelling older persons is expected to rise in line with the increase in longevity, placing increased burden on the individual, families and public health systems globally [4,8-10]. This highlights the need to identify potential clinical markers which may be able to identify individuals at risk of declining muscle function and falls so that appropriate prevention strategies can be instituted.

Osteocalcin (OC), the most abundant, non-collagenous bone protein, is synthesized and secreted by mature osteoblasts during bone formation and used clinically as a bone turnover marker [11,12]. OC exists in two major forms; γ-carboxylated (cOC) and undercarboxylated (ucOC) lacking γ-carboxylation at one or more sites [13]. cOC is thought to be predominately located in bone due to its high binding capacity to hydroxyapatite in-vitro, whereas ucOC was reported by some, but not all [14,15], to function in a paracrine and endocrine manner, participating in glucose metabolism and influencing muscle mass and strength. However, most of these data are limited to animal and preclinical studies [16-24].

We have previously shown that the ucOC/tOC ratio is higher in older compared to young adults [25], indicating that the ratio is a better measure of the aging effect on OC, than the total or individual forms separately. This, in part, could be due to lower intake of vitamin K, required for OC carboxylation [26]. Indeed, in older women, higher intake of leafy green vegetables, a rich source of vitamin K_1, was associated with greater muscle strength, improved muscle function and lower injurious falls risk, suggesting ucOC/tOC ratio may be involved, but this was not measured [27-29]. Therefore, whether ucOC or its ratio are possible candidates related to muscle function, and injurious falls risk remains to be fully elucidated.

As such, we tested the hypothesis in a large cohort of older women that a higher ratio of ucOC/tOC would be associated with reduced muscle function (strength and physical function) and an increased risk of long-term risk of falls-related hospitalizations.

4.3 Methods

4.3.1 Participants

The population included women recruited the Perth Longitudinal Study of Aging in Women (LSAW, http://www.lsaw.com.au/), the collective term referring to three studies across a 15-year period within the same population (1998-2013). Caucasian women were originally recruited to a 5-year, double-blind, randomized controlled trial of daily calcium supplementation to prevent fracture, the Calcium Intake Fracture Outcome Study (CAIFOS), described previously [30]. Women with an expected survival beyond 5 years and not receiving any medication (including hormone replacement therapy) known to affect bone metabolism were included. Women (n = 1500) were recruited from the Western Australian general population aged ≥70 years using the electoral roll. At the completion of the 5-year trial, women were invited to participate in two follow-up observational studies. Total follow-up was 14.5 years with baseline at 1998 (ending in 2013). Due to the link between vitamin D and falling [31], an additional 39 women were excluded as they were part of a sub study investigating calcium plus vitamin D supplementation. All participants provided written informed consent. Ethics approval was granted by the Human Ethics Committee of the University of Western Australia. Both studies were retrospectively registered on the Australian New Zealand Clinical Trials Registry (CAIFOS trial registration number #ACTRN12615000750583 and PLSAW trial registration number #ACTRN12617000640303) and complied with the Declaration of Helsinki. Human ethics approval for the use of linked data was provided by the Human Research Ethics Committee of the Department of Health, Western Australia (project number #2009/24).

4.3.2 Participant characteristics

Information regarding methodology of this trial has been previously published in detail [32]. In brief, information on medical history and current medications was obtained from the participant and then coded using the International Classification of Primary Care- Plus (ICPC-Plus) method [33]. The coding methodology allows aggregation of different terms for similar pathologic entities, defined by the ICD-10 coding system. Information about pre-existing diabetes was obtained from participants previous medical

history and current medications. Participants were asked to verify this information with their general practitioner, where available.

4.3.3 Body composition

Body weight was measured using digital scales to the nearest 0.1 kg and height assessed using a wall-mounted stadiometer to the nearest 0.1 cm. Participants were wearing light clothes and were without socks and shoes. Body mass index (BMI) (kg/m2) was then calculated. Whole body composition was measured at baseline or at 12 months by whole body dual-energy X-ray absorptiometry (DXA), using Hologic Acclaim QDR4500A dual energy X-ray absorptiometry machine (Hologic Corp., Waltham, MA) by several operators and analyzed according to a standard protocol. Each scan was reviewed by a supervisor for correct positioning. A calibration phantom was scanned at the beginning of each study participant session and evaluated using the Hologic provided Shewart rule program. CV s are under 2% in our laboratory. Appendicular lean mass was calculated as the sum of upper and lower limb mass (bone free).

4.3.4 Muscle parameters and physical activity levels

Grip strength was recorded from the dominant hand, recorded as the highest of 3 attempts [34] using a handheld dynamometer (hand Grip Dynamometer, TEC, Clifton, NJ). Physical function was measured by the timed up and go (TUG) test, where patients were timed to stand from the chair, walk 3 m, turn and then return to the seated position in the chair [35]. Participants performed one practice trial, before commencing the TUG test. Physical activity questionnaires were completed at baseline. Participants were asked about their participation in sport, recreation, and/or regular physical activities in the 3 months prior to their baseline visit. Previously described in detail [36,37] briefly, the level of activity, expressed in kilojoules per day, was calculated using a validated method applying activity type, time engaged in the activity, and the participant's body weight [38].

4.3.5 Estimation of dietary vitamin K

As vitamin K is related to the ratio of cOC to ucOC, we assessed dietary vitamin K intake at baseline for the previous 12 months using a validated, semi-quantitative food-frequency questionnaire (FFQ) [39]. The method has been previously described by our

group [40,41]. Briefly, total dietary vitamin K intake was calculated by multiplying the food items consumed (g/d) by the mean vitamin K value (μg/g). Vitamin K_1 (phylloquinone) values for FFQ food items were obtained from the US Department of Agriculture National Nutrient Database for Standard Reference (Release 28) [42]. Vitamin K_2 (menaquinone; MK-4 to MK-9) values for FFQ food items were obtained from Schurgers and Vermeer [43] and Vitamin K_2 (menaquinone; MK-10) values for FFQ food items were obtained from Manoury et al [44]. Where foods containing vitamin K were not available, a value of 0 μg/g was applied.

4.3.6 Biochemical measurements

The current post-hoc analysis (n= 1261) only included women with serum analyzed for tOC and ucOC from fasting blood samples collected in 1999, year-1 of the CAIFOS randomized controlled trial. Samples had not previously undergone a prior freeze-thaw cycle. An additional three participants with implausible ucOC/tOC ratio (> 1.0) were excluded. Serum tOC was measured by sandwich electrochemiluminescence immunoassay using the Roche Cobas N-Mid Osteocalcin assay (Roche Diagnostics, Mannheim). The inter-assay coefficient of variations (CV) were 2.3% and 4.8% at levels of 18 and 90ng/mL, respectively. Serum ucOC was measured by the same reagent assay with pre-treatment of the serum samples using 5mg/mL of hydroxyapatite (Calbiochem) following the method by Gundberg et al [13] and Chubb et al [45]. The inter-assay imprecision for percentage binding of cOC was 8% and 12% at OC concentrations of 100 and 15 ng/mL, respectively.

We assessed renal function as a possible confounder of OC as serum OC levels have been shown to be related to decreased renal clearance and chronic kidney disease (CKD) [46]. We used the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) creatinine-derived equation to estimate glomerular filtration rate (eGFR), we have described this method in detail previously [47].

Venous blood samples were collected between 08:30 a.m. and 10:30 a.m. after overnight fasting at baseline. Plasma was separated and stored in a –80°C freezer. Plasma 25OHD2 and 25OHD3 concentrations were determined using a validated liquid chromatography tandem mass spectrometry method at the RDDT Laboratories (Bundoora, VIC, Australia) according to published methodology [48] and summed to obtain a total plasma 25OHD concentration for each individual. Between-run coefficients

of variation (CVs) were 10.1% at a 25OHD2 mean concentration of 12 nmol/L and 11.3% at a 25OHD3 mean concentration of 60 nmol/L. An internal quality control (QC) test showed that the QC samples passed the acceptance criteria.

4.3.7 Falls outcomes

The method for assessing fear of falling was self-reported. Participants were asked to respond yes or no to a series of questions including "Are you afraid of falling?" "Do you limit any household activities because you are frightened you may fall?" and "Do you limit any outside activities because you are frightened you may all?". This method we have described in detail previously [36,49], briefly, a positive response to any of the three questions indicated a fear of falling.

Injurious falls (falls-related hospitalization) outcomes over 14.5 years were retrieved from the Western Australia Hospital Morbidity Data System via the Western Australian Data Linkage (Department of Health Western Australia, East Perth, Australia). Records were obtained for each of the study participants from 1998 until 2013 using the International Classification of External Causes of Injury codes and the International Classification of Diseases (ICD) coded diagnosis data pertaining to all public and private inpatient hospitalizations in Western Australia. This allows ascertainment of hospitalizations independent of self-report and avoids the problems of patient self-reporting and loss to follow-up. Falls from standing height or less, not resulting from external force were included (ICD-10 codes): W01, W05-W08, W10, W18, and W19. A fall was considered injurious if it required hospitalization. Prevalent self-reported falls were assessed by asking participants if they experienced a fall in the 3 months prior to their baseline clinical visit.

4.3.8 Blood pressure

Blood pressure was assessed in the morning after an overnight fast of at least 12 h, which included abstinence from tea, coffee and alcohol. A trained observer used a standard mercury sphygmomanometer to assess blood pressures. Participants rested in a seated position for a minimum of 5 min before blood pressure measurement commenced. Three blood pressures were then measured on the right arm at 1-min intervals. The mean blood pressure was calculated from these measurements.

4.3.9 Statistical methods

Our primary outcome was time to a falls-related hospitalization. Firstly, we explored whether the relationship between ucOC/tOC and falls-related hospitalizations was non-linear. As non-linearity was evident, we stratified participants into quartiles based on the ucOC/tOC ratio to explore characteristics of muscle function and other related variables. A one way ANOVA with Bonferroni correction or Mantel-Haenszel chi-squared difference in proportion was performed to determine significance between quartile groups. Non-parametric, Spearman rho' partial correlations were performed to determine associations of tOC, ucOC and the ucOC/tOC ratio using three different models of adjustment including Model 1: age and CAIFOS treatment (calcium supplementation versus placebo); Model 2: model 1 plus BMI, diabetes status, smoking history and prevalent atherosclerotic vascular disease (ASVD); and Model 3: model 2 plus eGFR. Prevalent ASVD was used as a possible confounder as we have shown previously in this cohort a link between ASVD prevalence and lower muscle strength [(50)]. For 5-years change in TUG and grip strength, these parameters were adjusted by the same models described, but in addition to the respective baseline measurement (not presented in table).

Cox-proportional hazards were used to examine the relationship between ucOC/tOC ratio and the time to first falls-related hospitalization in both unadjusted and multivariable-adjusted analysis. Global tests (estat phtest) indicated proportional hazards assumptions were not violated for all analysis (all $p>0.05$). The multivariable-adjusted model included: age, BMI, CAIFOS treatment, diabetes status, smoking history, and previous ASVD. The dose-response relationship between ucOC/tOC and falls-related hospitalizations were examined with penalized splines using the R package "survival" with df = 4 and using ucOC/tOC at 0.404 as the reference level, adjusted for all covariates as described in the multivariable-adjusted model. As poor vitamin D status is considered a risk factor for falls [(51)], we performed additional analyses where we included total 25OHD and season of blood sampling (as summer/autumn and winter/spring) to our multivariable-adjusted model. As blood pressure has the potential to influence falls risk, we performed additional analysis where we included mean systolic BP into our multivariable-adjusted model. Finally, we performed sensitivity analysis where muscle function measures were included in the multivariable-adjusted model when examining

the risk of falls-related hospitalization and ucOC/tOC. All statistics are reported in either mean ± SD, median and interquartile ranges or number and (%), and statistical significance accepted at $p<0.05$. Statistical analysis was performed with SPSS Statistics version 26 (IBM, USA), R statistics or Stata (version 13 StataCorp LP, College Station, TX).

4.4 Results

A total of 1261 women were included; the characterization of the women is presented in **Table 4.1.** Women were stratified into quartiles (**Table 4.2**) based on the ratio of ucOC/tOC (Q1 as the lowest ucOC/tOC group and Q4 as the highest) for the comparison of muscle strength, and functional measures between groups. Women with higher ucOC/tOC (Q3 and Q4) had a small, but significantly higher BMI (~1 $kg \cdot m^{-2}$) compared to women in Q1 and Q2 (all $p<0.05$). Women in Q4 had a slower time in TUG test (mean difference ~0.68 secs), compared to women in Q1 to Q3 ($p= 0.026$, 0.031 and 0.008, respectively, ANOVA $p=.004$). Walking aid use was significantly different across quartiles ($p= 0.012$). Fear of falling that limited house, outdoor and combined house and outdoor activities was significantly different across quartiles ($p= 0.026$, 0.044 and 0.017, respectively) (*Table 2*). There were no significant differences between quartiles for grip strength appendicular lean mass, METs or physical activity levels (all $p >0.05$), or 5-year change in TUG and grip strength (not presented in table, both $p >0.05$).

Table 4.1 Descriptive characteristics

Baseline characteristics	All participants (n= 1261)
Age (years)	75.22 ± 2.77
BMI (kg·m^{-2})	27.14 ± 4.65
Previous or current smoker (yes, %)	461 (36.6)
Diabetes (yes, %)	78 (6.2)
History of ASVD (yes, %)	149 (11.8)
Dietary calcium intake (mg/d)	956.81 ± 356.11 (n=1249)
METs, Kcal expended per day	111.55 (35.60-202.32)
Physically active (yes, %)	961 (76.2)
Grip strength (kg)	20.47 ± 4.56 (n= 1251)
Timed up and go (sec)	9.45 (8.18–11.11)
Appendicular lean mass (kg)*	14.85 ± 2.15
Fear of falling in house activities (yes, %)	214 (17.0)
Fear of falling in outdoor activities (yes, %)	184 (14.6)
Fear of falling in house and outdoor activities (yes, %)	130 (10.3)
Walking aid use (yes, %)	83 (6.6)
Dietary vitamin K intake (ug/d)	119.57 ± 46.56 (n= 1249)
tOC ng/mL	25.05 ± 10.28
ucOC ng/mL	11.99 ± 5.34
ucOC/tOC	.49 ± .12
eGFR CKD-EPI creatinine (1998)	66.25 ± 13.52 (n= 1135)

Results are mean ± SD, median and interquartile ranges or number and (%).

BMI, body mass index; ASVD, atherosclerotic vascular disease; METs, metabolic equivalents; tOC, total osteocalcin; ucOC, undercarboxylated osteocalcin; eGFR, estimated glomerular filtration rate; CKD-EPI chronic kidney disease epidemiological collaboration.

*Measured at either baseline or year

Table 4.2 Quartiles based on the ucOC/tOC ratio with measures of muscle function and falls parameters

	Quartile 1 (n= 315)	**Quartile 2** (n= 316)	**Quartile 3** (n= 315)	**Quartile 4** (n= 315)
Grip strength (kg)	20.59 ± 4.28	20.67 ± 4.29	20.54 ± 4.52	20.08 ± 5.10
Timed up and go (s)	9.22 (7.87-10.95)	9.33 (8.22-11.01)	9.30 (8.17-10.95)	**9.97 (8.55-11.53)[a,b,c]**
Appendicular lean mass (kg)* (n= 400)	14.52 ± 2.12 (n= 111)	14.84 ± 2.13 (n=94)	15.02 ± 2.16 (n=96)	15.07 ± 2.18 (n=99)
METs, Kcal expended per day	110.34 (44.78-209.76)	109.48 (38.09-203.42)	122.06 (0.00-216.99)	105.30 (0.00-181.03)
Physically active (yes, %)	249 (79.0)	243 (76.9)	237 (75.2)	232 (73.7)
Walking aid use (yes, %)	**15 (4.8)**	**17 (5.4)**	**21 (6.7)**	**30 (9.5)**
Fear of falling in house activities (yes, %)	**41 (13.0)**	**58 (18.4)**	**50 (15.9)**	**65 (20.6)**
Fear of falling in outdoor activities (yes, %)	**31 (9.8)**	**58 (18.4)**	**39 (12.4)**	**56 (17.8)**
Fear of falling in house and outdoor activities (yes, %)	**21 (6.7)**	**38 (12.1)**	**27 (8.6)**	**44 (14.1)**

Results are mean ± SD, median and interquartile ranges or number and (%). One way ANOVA with Bonferonni correction or Mantel-Haenszel chi-squared difference in proportion was performed to determine significance between groups. Bolded figures represent significant differences (P<0.05) across the quartiles by ANOVA or Mantel-Haenszel chi-square test of trend. [a] indicates significant difference (P<0.05) to quartile 1, [b] indicates significant difference (P<0.05) to quartile 2, [c] indicates significant difference (P<0.05) to quartile 3 after Bonferroni correction.

*Measured at either baseline or year 1.

Correlations between OC (tOC, ucOC and ucOC/tOC) with muscle function parameters were performed. A higher ucOC/tOC ratio was significantly related to poorer (slower) time to complete the TUG test (all adjusted models), but the correlation was weak (r range 0.06 to 0.1, p= .0001, .007 and .050, respectively). Grip strength was significantly but inversely correlated with ucOC (all adjusted models) however the relationship was weak (r = -.06, p= .033, .028 and .053, respectively). Five-years change score of the TUG (not presented in table) was significantly correlated with increased ucOC/tOC ratio in adjusted models 1 and 2, however this correlation was weak (r range .06 to .08, p= 0.010 and 0.032, respectively) and significance was lost when further adjusted for eGFR. A higher ucOC/tOC ratio was significantly correlated with walking aid use and fear of falling limiting household and combined house and outdoor activities using model 1; however, the correlation was weak (r range .06 to .09, p=.002, .024 and .041 respectively). Only walking aid use remained significantly correlated, although weak when adjusting for model 2 (r= 0.06, p=.034). Significance was lost when further adjusted for eGFR. There was no significant correlation for appendicular lean mass or 5-year change in grip strength under all models.

Penalized splines in **Figure 4.1** indicated a non-linear relationship between ucOC/tOC and the relative hazard for a falls-related hospitalization over 14.5 years. When looking at the relationship between ucOC/tOC and falls-related hospitalizations *(***Fig 4.2A and 2B***)* there was a non-linear association with Q1 and Q2 having similar risks and Q3 and Q4 having similar risks. As such, we combined Q1 and Q2 (referent group) and Q3 and Q4 (elevated ucOC/tOC ratio) for all further analyses. Kaplan Meier (unadjusted) and multivariable-adjusted Cox Survival curves by the median of ucOC/tOC are shown in **Figure 4.2A and 2B**, respectively (*see* ***supplementary figure 4.1*** *for these analyses by quartiles of ucOC/tOC*). Those with the above-median ucOC/tOC ratio had a higher relative hazard of falls-related hospitalizations (unadjusted log rank p=0.004) that remained significant in the multivariable-adjusted model HR 1.31, 95%CI 1.09-1.57, p= 0.004. In further adjustment analyses, we sought to determine the role of physical function (TUG) and grip strength in the relationship between ucOC/tOC and injurious fall risk. When adding muscle function (TUG and GS) to the multivariable adjusted model, this attenuated the relationship of ucOC/tOC with the relative hazard of a falls-related hospitalizations (HR 1.12 95%CI 0.99-1.28, p=.078). When comparing individuals with higher to lower ucOC/tOC, similar relative hazards were recorded for a falls-related hospitalization (HR 1.34 95%CI 1.13-1.62, p=0.002) when 25OHD (and season the

sample was obtained) was included in the multivariable-adjusted model. The inclusion of mean systolic BP to our multivariable-adjusted model did not augment the estimates for fall-related hospitalization when comparing women with higher to lower ucOC/tOC (HR 1.29 95%CI 1.07-1.54, p=0.007).

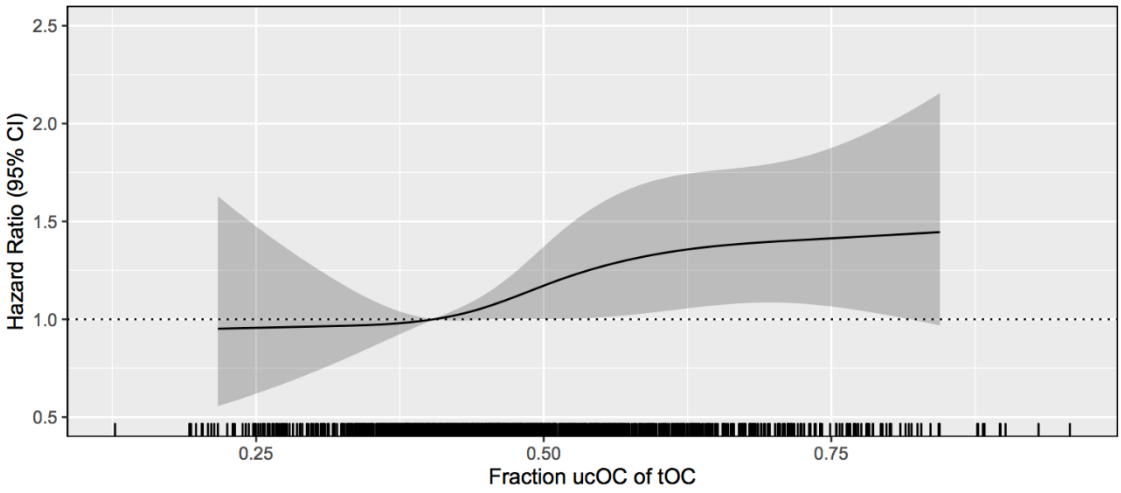

Figure 4.1 Multivariable-adjusted hazard ratios for ucOC/tOC ratio in relation to risk of a falls-related hospitalizations over 14.5 years based on fitted penalized splines using the median of Quartile 1 (0.40) as the reference level. The multivariable-adjusted model included age, BMI, treatment code (calcium/placebo), smoking history, ASVD history and diabetes (Model 2). Solid line is estimated HR and shaded areas represent 95% CI.

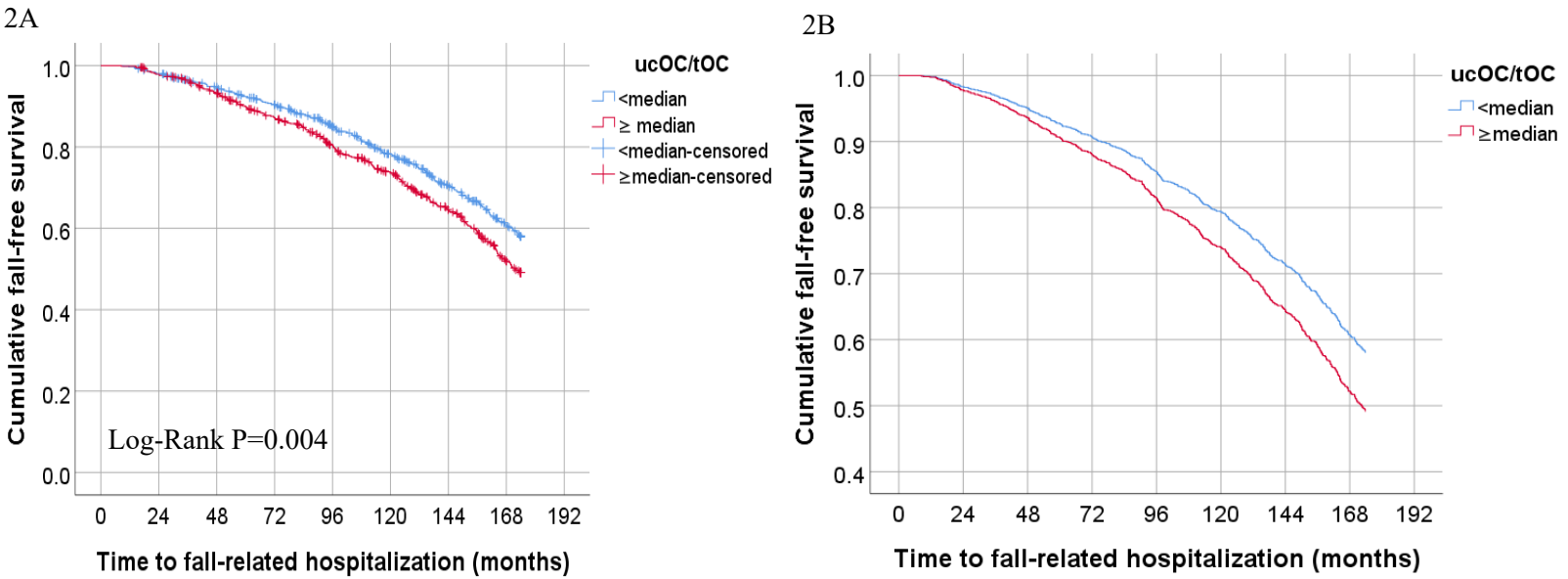

Figure 4.2 Kaplan-Meier curve (2a) and Cox regression analyses (2b) by the ucOC/tOC ratio and falls-related hospitalization. Kaplan Meier (unadjusted, log-rank p=0.004) and multivariable-adjusted Cox Proportional Hazards regression curves by the median of ucOC/tOC. Cox regression analysis adjusted for age, body mass index, treatment, diabetes, smoking history and previous atherosclerotic vascular disease.

4.5 Discussion

We report that in older women the ucOC/tOC ratio may be useful to identify a large proportion (~50%) of women with poorer TUG, including its decline, and increased risk for injurious falls requiring hospitalization. When comparing women based on quartile stratification of ucOC/tOC levels, we show that (a) those with the highest ucOC/tOC levels compared to those with the lowest, had the slowest TUG performance and, (b) with the ratio also sufficiently sensitive to detect fear of falling limiting home and outdoor activities suggesting falls may have already occurred. In addition, our results indicate that a higher ucOC/tOC ratio was significantly correlated to (c) poorer TUG performance, and worsening performance over a 5-year period and, (d) an increased risk of falls-related hospitalizations, even after adjustments of additional confounders. Finally, after adjustment for muscle function measures (TUG and GS), this relationship was attenuated suggesting the ucOC/tOC relationship with injurious falls may be via impaired muscle function.

Evidence from animal and preclinical studies demonstrate that ucOC may be involved in muscle maintenance, metabolism and strength, however, its function in humans is less clear [16,18-24]. We report that women with higher ucOC/tOC ratio have poorer physical function, mobility, and a greater fear of falling. We also demonstrate that physical function (TUG) is a key contributor to the relationship between the ucOC/tOC ratio and relative hazard of falls-related hospitalizations, suggesting that worsening function is associated with increasing ucOC/tOC ratio concomitantly increasing risk for falls. Previously we reported that higher ucOC/tOC ratio was related to increased muscle strength in older women [20]; however, only strength and not a physical function measure was assessed. A possible explanation for the conflicting results is the fasting/feeding state at the time of the blood sampling. In both studies blood was collected in the morning, however, in the current study women fasted overnight prior to blood collection, whereas in the previous study blood was sampled following a light meal. We and others have previously demonstrated that a meal and/or glucose load can suppress OC and ucOC, and other bone markers [52-55]. Therefore, this may affect the correlations and it may also make it difficult to compare between these studies. Notably, similar results from both studies show that the ucOC/tOC ratio is potentially a more sensitive measure, than ucOC alone, as an indicator of physical function and possibly falls-related clinical outcomes, at least in humans.

Clinically, circulating tOC levels are used as a surrogate measure for bone turnover [11,12,56-58], and we have shown that aging is associated with a "U"-shape pattern across the adult lifespan for all OC-forms in men [25]. Currently, only tOC is measured clinically, and in research however, emerging evidence, including this work, suggests that more could be understood about the dynamics of OC and its forms and the relationships to clinical outcomes. We demonstrate in the current study that older women with higher ucOC/tOC ratio, which would suggest poorer vitamin K status, have greater risk of falls-related hospitalizations. This is of clinical relevance as poorer vitamin K status could be detrimental. Notably, higher vitamin K intake has been associated with improved muscle function, a reduction in falls and improved bone quality, possibly also decreasing fracture risk [27,28,40]. Overall, our findings are in agreement with others, who show that lower cOC/tOC (or conversely, higher ucOC/tOC) can predict fracture risk in older men [59] but adds a further potential mechanism, poor muscle and physical function. In older women, it appears that the ratio is more strongly related to physical function and falls risk than ucOC alone.

The findings of the current study may partly be explained by age-related changes on the skeleton and skeletal system in an attempt to maintain normal bone homeostasis. It is possible that the increase in ucOC with ageing is related to the deterioration in the material property of bone as we age. Other potential compensatory mechanisms may be explained by the weakening muscle potentially altering osteoblast physiology perhaps via a biochemical or biomechanical cross-talk, as an attempt to stimulate the failing osteoblasts [60,61]. Another possible explanation could be related to the increasing vitamin K requirement as we age to maintain both bone and muscle health [27,28,40,62,63]. Interestingly in our cohort, women with higher ucOC/tOC levels and poorer muscle function parameters also had lower dietary vitamin K intake and, their ucOC/tOC levels would fall in the upper limit of our previously proposed thresholds limits for older men of similar age [25]. We now extend on our previous work by demonstrating that a higher ucOC/tOC ratio, in older women, is related to poorer TUG performance, and an increased risk for falls-related hospitalization, partly explained by impaired muscle function. This suggests that the ucOC/tOC ratio may enable, in the clinical setting, the early identification of individuals at risk for low physical function and prevent future falls.

4.5.1 Strengths

The strengths of our study include that we have investigated, for the first time, the longitudinal relationship between ucOC/tOC ratio with muscle function and falls risk in a large cohort of older women. All samples in this study were obtained in a fasted state and in the morning, which accounts for the known effects of the circadian rhythm on OC [52-54]. This is also the first study to investigate the relationship of OC, ucOC and the ucOC/tOC ratio with indices of muscle function and, between the ucOC/tOC ratio and time to falls related hospitalization data. In addition, all OC analysis was completed by the same technician and laboratory utilizing identical methodology, thus minimizing variation due to technical error.

4.5.2 Limitations

There are some potential limitations of our study. While the data set is large, it only includes data consisting of predominately Caucasian older women, therefore our findings may not be generalizable to other ethnicities, younger individuals, or men. Additionally, we cannot infer causality based on our results due to the observational nature of the study design. We have used the methods proposed by Gundberg et al. (467) to analyze ucOC. However, the levels are highly dependent on the technical details including the antibody, specific binding capacity of the hydroxyapatite, amount of the apatite or ELISA used. Different research groups also use different methodologies to analyze ucOC and therefore the findings of this study may not be transferable to studies that have used different techniques. Future studies should explore the relationship of OC, ucOC and the ucOC/tOC ratio with muscle parameters and falls outcomes in men as this may be different than observed for women. A further limitation is medications known to increase falls risk such as sedatives, anti-epileptic drugs and antidepressants being taken at the baseline visit were not available for this study. Lastly, it will be important that future studies will independently replicate our findings to provide further evidence for the use ucOC/OC to identify older women at higher risk of falls-related hospitalization.

4.5.3 Conclusion

In summary, increased ucOC/tOC ratio is associated with low physical function and increased risk of falls related hospitalization in older women. Early identification of

women at higher risk may enable prevention and intervention strategies to occur, reducing risk for injurious falls.

4.6 References

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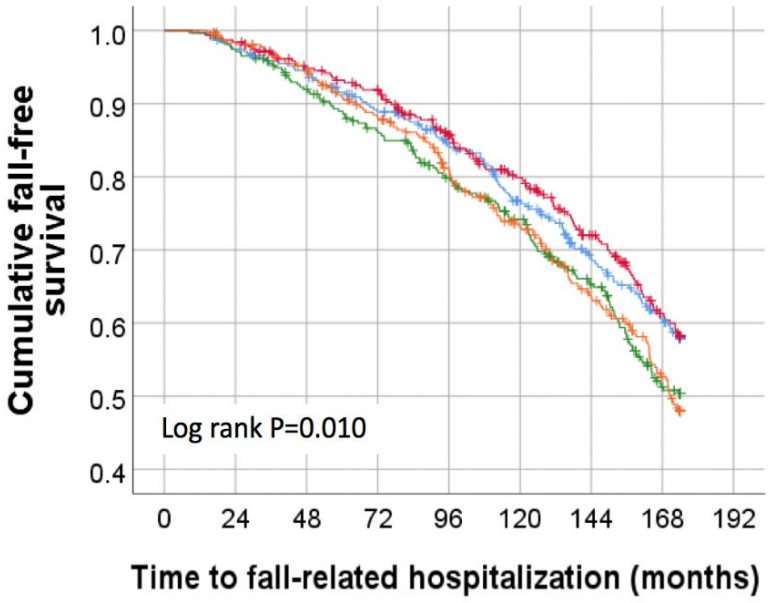
4.7 Supplementary materials

2C



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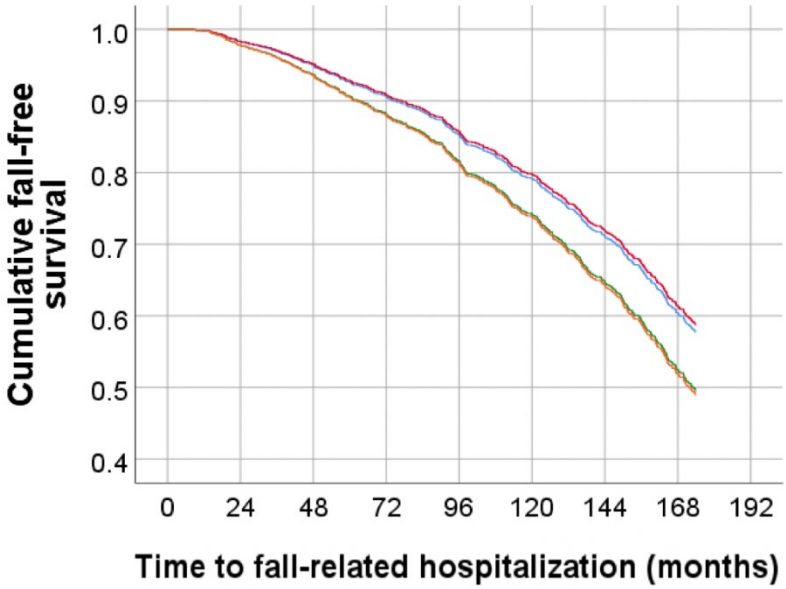

Supplementary figure 4.1 Kaplan Meier (A) (unadjusted, log-rank p=0.010) and multivariable-adjusted Cox Proportional Hazards regression curves (B) by quartiles of ucOC/tOC. Adjusted for age, body mass index, treatment, diabetes, smoking history and previous atherosclerotic vascular disease.

Chapter 5: The effects of acute exercise on bone turnover markers in middle-aged and older adults: a systematic review

Context:

Available evidence demonstrates that acute exercise can alter BTMs, yet majority of studies are performed in younger adults. It is also unclear if different modes of exercise affect BTMs differently. Here, we systematically reviewed the literature for acute exercise studies investigating BTMs in middle aged and older adults. This study was a crucial first step to understanding if older adults respond differently to younger adults, and if other characteristics of the exercise protocol effect BTMs differently. This was also an important precursor step prior to developing the clinical trial in study 4.

This study has been published:

Smith, C., Tacey, A., Mesinovic, J., Scott, D., Lin, X., Brennan-Speranza, T. C., Lewis, J. R., Duque, G., & Levinger, I. (2021). The effects of acute exercise on bone turnover markers in middle-aged and older adults: A systematic review. *Bone*, *143*, 115766. https://doi.org/10.1016/j.bone.2020.115766

Please see **Appendix 4** for the published version of this manuscript.

I have also presented this study at two conferences:

- Australia and New Zealand Bone and Mineral Society conference (2021) as a poster and e'poster
- World Confress of Osteoporosis, Osteoarthritis and Musculoskeletal disease-International Osteoporosis Foundation Conference (WCO-IOF-ESCEO) (2021) as a poster and e'poster

OFFICE FOR RESEARCH TRAINING, QUALITY AND INTEGRITY

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

1. PUBLICATION DETAILS (to be completed by the candidate)

Title of Paper/Journal/Book: The effects of acute exercise on bone turnover markers in middle-aged and older adults: A systematic review

Surname: Smith First name: Ca

Institute: Institute for Health and Sport Candidate's Co

Status:

Accepted and in press: ☐ Date:

Published: ☑ Date: 21/1

2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be inclu ed in the HDR Policy and related Procedures – policy.vu.edu.au.

Cassandra Smith Digitally signed by Cassandra Smith Date: 2022.03.19 11:24:21 +11'00' | 19/03/2022

Signature **Date**

3. CO-AUTHOR(S) DECLARATION

In the case of the above publication, the following authors contributed to the w

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in t or interpretation of at least that part of the publication in their field of e
2. They take public responsibility for their part of the publication, excep nor who accepts overall responsibility for the publication;

3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to a) granting bodies, b) the editor or publisher of journals or other publications, and c) the head of the responsible academic unit; and
5. The original data will be held for at least five years from the date indicated below and is stored at the following **location**(**s**):

Victoria University

Name(s) of Co-Author(s)	Contribution (%)	Nature of Contribution	Signature	Date
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Jakub Mesinovic	10	Da editing of t		22/03/2022
David Scott	5	uscript		21/03/2022
Xuzhu Lin	2.5	uscript		22/03/2022
Tara C. Brennan-Speranza	2.5	uscript		22/03/2022
Joshua R. Lewis	5	uscript		21/03/2022
Gustavo Duque	5	uscript		23/03/2022

The effects of acute exercise on bone turnover markers in middle-aged and older adults: a systematic review

Running heading: Acute-exercise and bone turnover markers in middle-aged and older adults

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5.1 Abstract

Background: Bone turnover is the cellular machinery responsible for bone integrity and strength and, in the clinical setting, it is assessed using bone turnover markers (BTMs). Acute exercise can induce mechanical stress on bone which is needed for bone remodelling, but to date, there are conflicting results in regards to the effects of varying mechanical stimuli on BTMs.

Objectives: This systematic review examines the effects of acute aerobic, resistance and impact exercises on BTMs in middle and older-aged adults and examine whether the responses are determined by the exercise mode, intensity, age and sex

Methods: We searched PubMed, SCOPUS, Web of Science and EMBASE up to 22nd April 2020. Eligibility criteria included randomised controlled trials (RCTs) and single-arm studies that included middle-aged (50 to 65 years) and older adults (>65 years) and, a single-bout, acute-exercise (aerobic, resistance, impact) intervention with measurement of BTMs. PROSPERO registration number CRD42020145359

Results: Thirteen studies were included; 8 in middle-aged (n= 275, 212 women/63 men, mean age= 57.9 ± 1.5 years) and 5 in older adults (n= 93, 50 women/43 men, mean age= 68.2 ± 2.2 years). Eleven studies included aerobic exercise (AE, 7 middle-aged/4 older adults), and two included resistance exercise (RE, both middle-aged). AE significantly increased C-terminal telopeptide (CTX), alkaline phosphatase (ALP) and bone-ALP in middle-aged and older adults. AE also significantly increased total osteocalcin (tOC) in middle-aged men and Procollagen I Carboxyterminal Propeptide and Cross-Linked Carboxyterminal Telopeptide of Type I Collagen in older women. RE alone decreased ALP in older adults. In middle-aged adults, RE with impact had no effect on tOC or BALP, but significantly decreased CTX. Impact (jumping) exercise alone increased Procollagen Type 1 N Propeptide and tOC in middle-aged women.

Conclusion: Acute exercise is an effective tool to modify BTMs, however, the response appears to be exercise modality-, intensity-, age- and sex-specific. There is further need for higher quality and larger RCTs in this area.

5.2 Introduction

The skeleton has protective, mechanical and metabolic roles, providing structural support and a site for calcium storage (1-3). Bone should be strong, to prevent fractures, but light, to enable movement in a gravitational environment (1). Bone turnover, the cellular machinery responsible for bone integrity and strength, is a finely balanced process responsive to mechanical loads and hormonal changes (4-6).

Exercise is a non-pharmacological intervention that can improve bone health and reduce the risk of osteoporosis (7-11). The anabolic effects of exercise on osseous tissues are positively associated with the amount of mechanical strain exerted (12). In animals, the strain-adaptive remodelling response requires intermittent and dynamic, but not static, loading (13-18). Additionally, loading periods only need to be very short to stimulate adaptive responses, and that bone formation is threshold-driven and influenced by strain rate, frequency, amplitude and duration of loading (17-22). Altogether, these findings demonstrate that bone requires dynamic (not static) strains (i.e. impact loading) for adaptive responses and, higher physiological rates compared to low rates and applied rapidly, to increase this response (14-16, 19, 23).

In humans, higher impact activities with rapid rates of loading (i.e. tennis/squash) are more osteogenic compared with lower impact sports (i.e. running/cycling) (24-26). Mechanical loads, produced by exercise, change local microenvironments of the canalicular networks within the bone framework via dynamic fluid shifts stimulating local osteocytes and ultimately bone turnover (27-29). Exercise serves varying purposes across the lifespan. In children, exercise is important for optimisation of peak bone mass, whereas, in older adults, exercise serves to maintain/reduce the rate of bone loss (9, 10, 30). However, the search for a relationship between exercise and bone mineral density (BMD) demonstrates contradictory findings, some reporting beneficial effects (7, 11, 31), while others have not (32-34). Moreover, available human data shows that the magnitude of benefit on bone from exercise is inconsistent, often influenced by safety concerns leading to conservatively prescribed training loads (35-39).

To optimise exercise effects on bone health a better understanding of the metabolic responses of bone tissue to various mechanical stimuli is needed. By convention, BMD is widely used as a measure of bone health to predict fracture risk (40), however, it represents a static bone mineral status and cannot be used to estimate acute bone metabolic changes such as those induced by acute exercise. Therefore, BTMs

represent an easy to measure option to assess the dynamic fluctuations in bone turnover (*Table 1*) (41). Using BTMs to describe bone metabolic activity comes with complexities, contributing to the lack of consensus in the literature. Whilst these markers are sensitive, they have high biological variability attributed to differences in i.e. blood sampling, study protocols, effects of feeding and circadian rhythm (41-43). As such, the aims of this systematic review were to 1) examine the effects of acute exercise on BTMs in adults over 50 years of age and to determine if middle-aged and older adults respond differently, and 2) to understand whether these effects were exercise modality-, exercise intensity-, sex- or BTM-specific.

Table 5.1. Markers of bone turnover that have been used in the exercise literature

Markers of bone resorption	
C-terminal crosslinked telopeptide of type I collagen	CTX, Crosslaps
N-terminal telopeptide of type I collagen	NTX
Cross-linked carboxyterminal telopeptide of type I collagen	ICTP
Tartrate-resistant acid phosphotase	TRAP
Receptor activator of nuclear factor κB ligand	RANKL
Sclerostin	SCL
Markers of bone formation	
Alkaline phosphatase (total)	ALP
Alkaline phosphatase (bone specific)	B-ALP
Procollagen I carboxyterminal propeptide	*PICP*
Procollagen type 1 n propeptide	*P1NP*
Osteocalcin	OC
Osteoprotegerin	OPG

5.3 Methods

This systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines (44) and was registered in the International Prospective Register of Systematic Reviews (PROSPERO) - CRD42020145359.

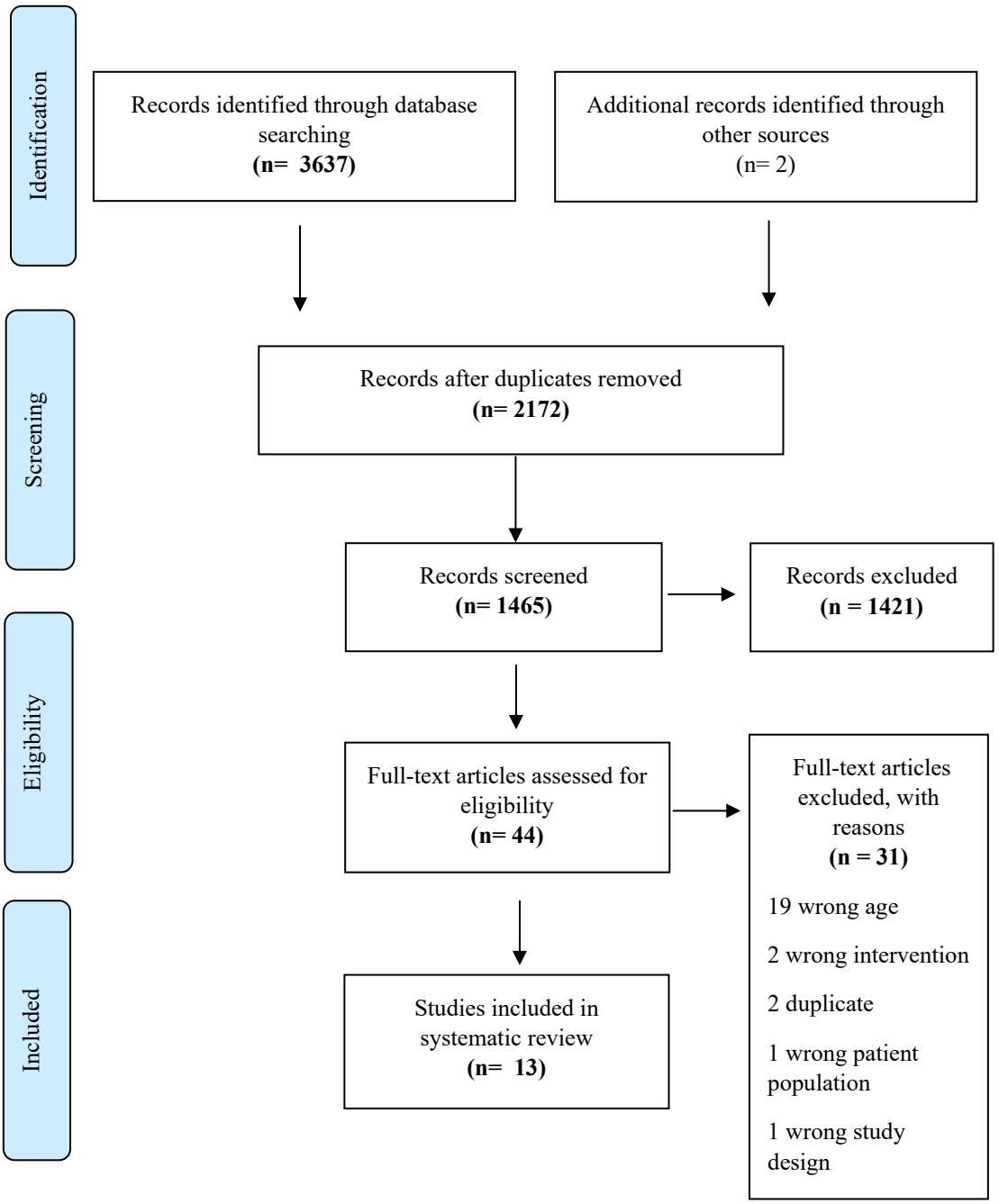

Figure 5.1 Identification screening and selection of studies (PRISMA Flow Diagram)

5.3.1 Inclusion criteria

The inclusion criteria for studies in brief were: (i) randomised controlled (RCT), cross-sectional or single arm trials including quasi-randomised design; (ii) adults ≥50 years of age, middle-aged adults defined as mean age ≥50 to <65 years and older adults defined as mean age ≥65 years; (iii) intervention of interest includes acute bout or single-bout of exercise; and (v) outcome of interest was BTMs (*see* ***supplementary 5.1, PICOS protocol***).

5.3.2 Data extraction

CS and AT performed the literature search (***supplementary 5,2****, search strategy*) and extracted data from the included studies, IL revised discrepancies. The following data were extracted: (i) characteristics of the participants i.e. sample size, sex, age (years), height (centimetres), weight (kilograms) and body mass index (BMI, height/weight^2); (ii) details of the acute exercise intervention (intensity, duration, volume, mode); and (ii) details of outcomes of interest (BTMs) measured at baseline and post- acute exercise.

5.3.3 Quality assessment: Risk of bias and Methodological Index for Non-Randomised Studies

Risk of bias assessments were independently conducted by CS and AT. RCTs were assessed using the Cochrane Collaborations Risk of Bias 2 (ROB2) tool (45). We assessed selection bias (random sequence generation, allocation concealment), performance bias (blinding of participant and personnel), detection bias (outcome assessor blinding), attrition bias (handling of incomplete outcome data) and other bias including baseline imbalance on the primary outcome and selective reporting. All other trials not meeting the criteria for a RCT were assessed using the Methodological Index for Non-Randomised Studies (MINORS) scale (46).

5.4 Results

We identified 3637 articles. After removal of duplicates, 1465 unique titles and abstracts were screened, and 1421 articles were excluded. The full text of 44 articles was reviewed and a further 31 were excluded, leaving 13 articles for inclusion in our qualitative synthesis (***Fig. 5.1)***. The authors of four studies were contacted for further information (47-50). One intervention was described in two articles but with different stratification of groups, both articles were included and considered as a single trial (51, 52). Another study had additional analyses published at a later date, both articles were included but considered as a single trial (49, 50). Herein for both of these studies, the first published paper will be referenced.

5.4.1 Quality assessment

Results of the methodological quality assessments are shown in ***Table 5.2*** and ***Figure 5.2***. Only 3 studies were RCTs (53-55) and assessed using the ROB2 tool. All others were assessed using the MINORs scale. No studies achieved a maximum quality score. Scores ranged on the ROB2 (***Figure 5.2)*** and on the MINORs scale (***Table 5.2***) from 43.8% to 87.5%. The most common source of likely methodological bias using the ROB2 tool was the randomisation process and deviations from the intended study endpoint. Using the MINORs scoring system, the likely source of methodological bias was the absence of unbiased assessment of the study endpoint (n= 10) and prospective calculation of study sample size (n= 8).

Table 5.2. Quality rating scale (MINORs)

MINORs Scale (detailed below and scored as: 0, not reported; 1, reported but inadequate; 2, reported and adequate) Field 9 to 10 only relevant for comparative studies													
Author, year	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	Score
1. Kim, et al. (2014)	1	1	0	1	0	2	2	0	n/a	n/a	n/a	n/a	43.8 %
2. Levinger, et al. (2011)	2	2	2	2	0	2	2	0	1	2	2	2	79.2 %
3. Levinger, et al. (2014)	2	2	2	2	0	2	2	2	n/a	n/a	n/a	n/a	87.5 %
4. Maimoun, et al. (2005)	1	2	2	2	0	2	2	0	1	1	2	2	70.8 %
5. Rudberg, et al. (2000)	2	1	0	2	0	2	2	0	n/a	n/a	n/a	n/a	56.3 %
6. Thorsen, et al. (1995)	2	1	1	2	0	2	2	0	n/a	n/a	n/a	n/a	62.5 %
7. Thorsen, et al. (1996)	2	1	1	2	0	2	2	0	n/a	n/a	n/a	n/a	62.5 %
8. Aly, et al. (2017)	1	1	2	2	0	2	2	0	n/a	n/a	n/a	n/a	62.5 %
9. Wherry, et al. (2019)	2	2	2	2	0	2	2	2	1	2	2	1	83.3 %
10. Zerath, et al. (1997)	2	1	2	2	0	2	2	0	n/a	n/a	n/a	n/a	68.8 %

MINORs Scale assessed as per; 1. A clearly stated aim; 2. Inclusion of consecutive patients; 3. Prospective data collection; 4. Endpoints appropriate to study aim; 5. unbiased assessment of study endpoint; 6. follow up period appropriate to the aim; 7. loss to follow up < 5%; 8. Prospective calculation of study size; 9. adequate control group; 10. contemporary groups; 11. Baseline equivalence of groups and 12. Adequate statistical analysis

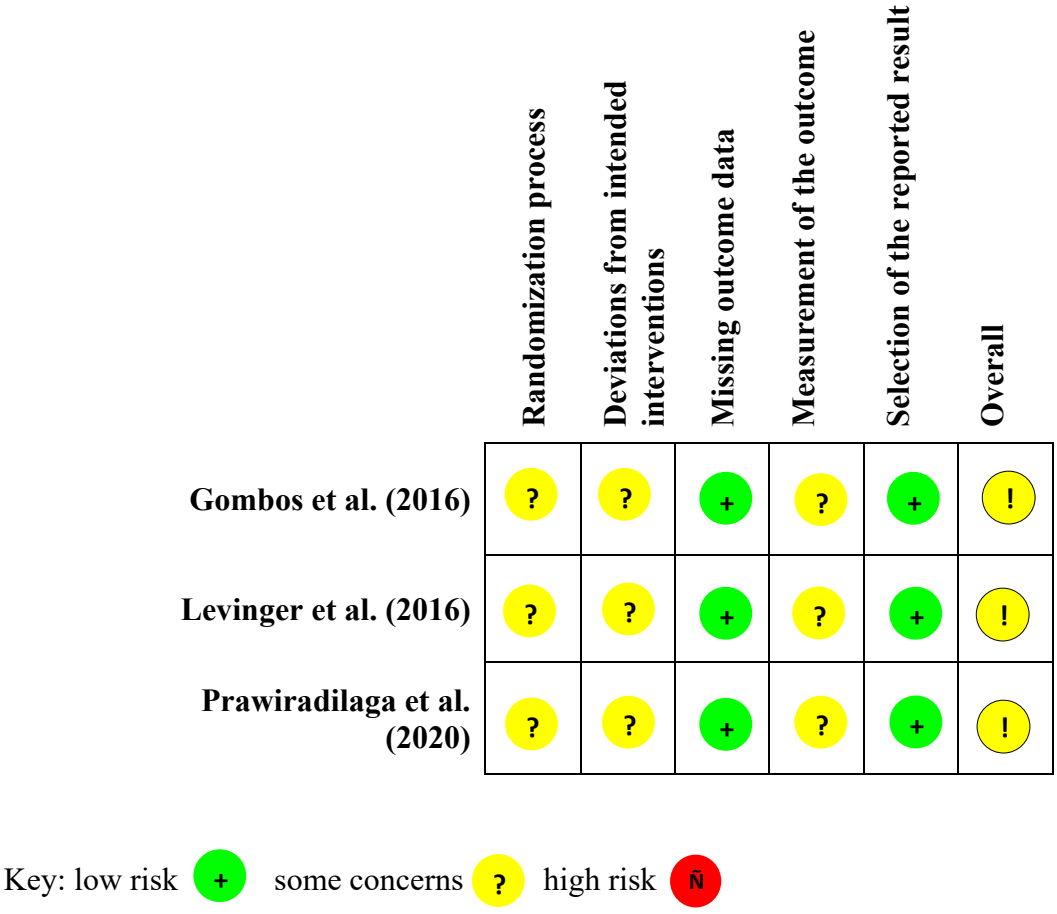

Figure 5.2 Risk of bias ratings

5.4.2 Study population and study design

Descriptive characteristics and study outcomes of included studies are described in ***Table 5.3.*** Two studies included adults with osteoporosis (untreated) (54, 56), five studies excluded individuals with osteoporosis/conditions affecting bone metabolism (48, 49, 52, 53, 57) and one study included adults with osteopenia (47). Four studies did not state whether they excluded participants with osteoporosis (55, 58-60). Five studies excluded individuals taking medications/supplements that effect bone metabolism (47, 49, 52, 53, 57), one stated except for calcium and vitamin D (54), four studies included participants not taking medications (56, 58, 60, 61) and three studies did not refer to medication use (48, 55, 59).

Of the thirteen studies included, eight were in middle-aged (mean age <65 years) (48, 49, 53-55, 58-60) and five were in older adults (mean age >65 years) (47, 52, 56, 57,

61). Sample sizes ranged from 11 to 150 (total combined data of the 13 studies n= 336 [220 women, 116 men]). Participants' age range was 52 to 73 years (mean age 62 ± 6 years) and BMI was 23.5 to 33.1 kg/m^2 (mean BMI 26.85 ± 3.33 kg/m^2). Sex-distribution for included studies was predominately women (71%); 77% of middle-aged and 54% of older adults were women.

Eleven studies evaluated effects of acute AE exercise on BTMs (seven in middle-aged (48, 49, 54, 55, 58-60), and four in older adults (47, 52, 57, 61)). Two studies evaluated effects of acute combined RE and impact (middle-aged adults) (48, 54), one study evaluated the effects of acute impact exercise alone (middle-aged adults) (53), and one study evaluated the effects of acute RE alone (older adults) (56) on changes in BTMs. Only two studies reported that the exercise was supervised (47, 53). Exercise protocols, blood sampling protocols and effects of acute exercise on BTMs have been described in *Table 3* including all reported levels and significant changes.

Nine studies reported that exercise and blood sampling were performed in the morning (48, 49, 52-55, 58, 59, 61), one was performed in the afternoon (60), and three did not state the time of the day (47, 56, 57). Seven studies were performed in the morning following an overnight fast (48, 49, 52-55, 59), one stated at least 12-hours of fasting (no indication of time) (56), and five studies were not performed in a fasted state (47, 57, 58, 60, 61). One study involved a controlled pre-feed (47), and another stated a 2-hour fast after a meal free from milk and cheese (60). Only three studies reported controlling for exercise on preceding days (53, 58, 61). One study mentioned withholding dietary supplements (53). Post-exercise blood sampling varied greatly from one to four timepoints; four studies taking only immediately post (51, 52, 54, 57, 59), the longest taken at 72-hours (58, 61).

Table 5.3. Study characteristics and outcomes

Study details Author, journal (year)	**Study design**	**Participants** Sample (n, M/F) Age (years) Height (cm) Weight (kg) or BMI (kg/m^2) mean ± SD	**Acute exercise intervention** T: type D: duration I: intensity S&R: sets and reps	**Blood sampling protocol** T: timepoints C: controls B: BTMs measured	**Main findings** Effects of acute exercise compared to baseline
Middle-aged adults mean age 50 to 65 years					
Gombos et al. BMC Musculoskeletal Disorders (2016) (54)	RCT	**Healthy middle-aged women** n= 150 **RE + IMP *(n=50)*** 60.2 ± 6.9 yrs 162.6 ± 9.8 cm 69.7 ± 11.8 kg 26.3 ± 5.4 kg/m^2 -2.2 ± 0.7 T-score ***AER (n=50)*** 58.7 ± 6.3 yrs 159.6 ± 6.4 cm 72.7 ± 14.8 kg 27.2 ± 6.1 kg/m^2 -1.9 ± 0.9 T-score ***CON (n=50)*** 57.8 ± 8.4 yrs	Randomised to: **1. RE + IMP** T: resistance exercises of large muscle groups, core stabilisation and impact D: 5 mins warm up, 30 mins resistance exercises, 8 mins cool down I: not stated S&R: 3 sets of 4 to 8 reps **2. AER** T: brisk walking (W) at 100 steps/min D: 46 mins I: moderate intensity at 3 to 6 METs **3. CON** T: nil intervention	T: baseline, post ex (+0 to 5 min) C: OFT, AM B: CTX, BALP and SCL	**Post exercise at 0 to 5 min** (all mean ± SD) ↑ BALP AER only *RE+IMP 41.7 ± 12.8 to 41.8 ± 12.0 %* *AER 41.8 ± 7.6 to 42.1 ± 8.4 % ** *CON 42.2 ± 10.4 to 42.1 ± 10.2 %* ↓ CTX RE+IMP only *RE+IMP 303.6 ± 156.8 to 276.4 ± 143.6 pg/mL*** *AER 247.3 ± 106.2 to 253.9 ± 107.5 pg/mL* *CON 259.1 ± 110.2 to 256.7 ± 111.2 pg/mL* ↑ SCL AER only *RE+IMP 26.8 ± 14.0 to 29.8 ± 15.7 pmol/L* *AER 23.6 ± 10.0 to 29.9 ± 10.8 pmol/L*** *CON 24.0 ± 8.8 to 24.2 ± 8.8 pmol/L*

Study details Author, journal (year)	**Study design**	**Participants** Sample (n, M/F) Age (years) Height (cm) Weight (kg) or BMI (kg/m^2) mean ± SD	**Acute exercise intervention** T: type D: duration I: intensity S&R: sets and reps	**Blood sampling protocol** T: timepoints C: controls B: BTMs measured	**Main findings** Effects of acute exercise compared to baseline
		161.7 ± 5.0 cm 69.5 ± 13.0 kg 28.1 ± 3.9 kg/m^2 -2.1 ± 0.7 T-score *(T-score site not stated)*			
Levinger, I., et al. Osteoporos Int (2011) (48)	Randomised parallel design	**Middle-aged obese, men** n= 28 ***AER (n= 13)*** 52.8± 5.41 yrs 174.9 ± 6.49 cm 100.5 ± 18.75 kg 32.7 ± 5.41 kg/m^2 **RE + IMP *(n= 15)*** 52.1 ± 6.97 yrs 177.7 ± 5.03 cm 99.2 ± 13.94 kg 31.5 ± 4.65 kg/m^2	Randomised to: **1. AER** T: cycling D: 45 mins I: 75% of VO^2_{Peak} **2. RE + IMP** T: resistance exercise including power leg press and jumping D: 45 mins I: 70 to 75% of 1RM S&R: 2 x 5 sets of 8 leg press, 3 x 5 sets of 10 jumps	T: baseline, post ex (+0, 30, 60 and +120 min) C: OFT, AM B: tOC (and ucOC)	**Post exercise to peak** (all mean ± SD) ↑ tOC AER group only *AER 5.32 ± 2.89 to 6.08 ± 3.51 ng/mL* ** *RE+IMP 4.82 ± 1.63 to 5.01 ± 2.03 ng/mL* ↑ ucOC AER group only *AER 4.64 ± 3.03 to 5.08 ± 3.5 ng/mL* ** *RE+IMP 3.93 ± 1.53 to 3.99 ± 1.51 ng/mL*

Study details Author, journal (year)	**Study design**	**Participants** Sample (n, M/F) Age (years) Height (cm) Weight (kg) or BMI (kg/m^2) mean ± SD	**Acute exercise intervention** T: type D: duration I: intensity S&R: sets and reps	**Blood sampling protocol** T: timepoints C: controls B: BTMs measured	**Main findings** Effects of acute exercise compared to baseline
Levinger, I., et al. JBMR (2014) (49) additional analysis for PINP and β-CTX reported in **Levinger, I., et al**. BoneKEy Rep (2015) (50)	Non-randomised, case-controlled crossover	**Middle-aged obese, non-diabetic men** n= 11 58.1 ± 7.29 yrs 176 ± 5.64 cm 102.5 ± 12.93 kg 33.1 ± 4.64 kg/m^2	Completed both **1. CON** T: complete rest D: 30 mins **2. AER** T: cycle ergometer, high intensity exercise D: 30 mins I: 4 min warm up @ 50 to 60 % HR_{Peak} & cycling as: 4 x 4min @ 90 to 95% HR_{Peak} 2 min active recovery @ 50 to 60% HR_{Peak}	T: baseline, post ex (+0, 30 and 60 min) C: OFT, AM B: tOC (and ucOC, ucOC/tOC) and P1NP and β-CTX	**Post exercise to peak** (all mean ± SEM) NC tOC *AER 18.2 ± 1.4 to 18.61 ± 1.48 ng/mL* NC P1NP *AER 36.1 ± 1.3 to 37.09 ± 1.56 μ/L^{-1}* ↑ β-CTX (~16%) *AER 306.5 ± 41 to 357.45 ± 50.33 μ/L^{-1}* ** ↑ ucOC (~2.1%) *AER 10.6 ± 0.8 to 11.21 ± 0.69 ng/mL* * ↑ ucOC/OC (~1.9%) *AER 58.9 ± 2.0 to 62.1 ± 1.9 %* *
Levinger, I., et al. Physiol Rep (2016) (55)	Randomised, case-controlled crossover	**Postmenopausal women** n= 10 62.8 ± 8.22 yrs 161.2 ± 5.06 cm 73.6 ± 10.75 kg 28.3 ± 4.11 kg/m^2	Completed both: **1. CON** T: complete rest D: 30 mins *2***. AER** T: cycle ergometer	T: baseline, post ex (+0, 30, 60 and 120 min) C: OFT, AM B: β-CTX, P1NP, tOC (and ucOC)	**Post exercise to peak** (all mean ± SD) NC tOC *28.1 ± 8.6 to 28.38 ± 8.76 ng/mL* NC P1NP 67.2 ± *7.6 to 62.6 ± 19.09 μ/L^{-1}*

Study details Author, journal (year)	**Study design**	**Participants** Sample (n, M/F) Age (years) Height (cm) Weight (kg) or BMI (kg/m^2) mean ± SD	**Acute exercise intervention** T: type D: duration I: intensity S&R: sets and reps	**Blood sampling protocol** T: timepoints C: controls B: BTMs measured	**Main findings** Effects of acute exercise compared to baseline
			D: 30 mins I: 70 to 75 % of VO^2_{Peak}		NC β-CTX 429.3 ± *40.1 to 470.1 ± 145.61 μ/L^{-1}* ↑ ucOC *13.83 ± 6.71 to 15.04 ± 7.35 ng/mL* **
Prawiradilaga et al. Biol Sport (2020) (53)	RCT-crossover	**Healthy, sedentary postmenopausal women** n= 29 60.0 ± 5.6 yrs 165.2 ± 5.4 cm 65.8 ± 7.7 kg 24.1 ± 2.5 kg/m^2	Each participant performed in a random order 3 high-impact exercise trials and CON **Session 1- IMPACT** T: 7 min low impact warm up on a gymnastic mat, then counter movement jump (CMJ) vertical jump with two leg launch and land. **Session 2- IMPACT** T: 7 min low impact warm up on a gymnastic mat then drop jump (DJ) from a 32 cm box, the landing continued into a vertical two-leg jump	T: baseline, post ex (immediately after and +2 hrs) C: AM, OFT, nil vigorous exercise preceding 48 hrs, dietary supplements withheld B: P1NP, tOC, CTX	**Post exercise at 0 min** (all mean ± SE) P1NP ↑ for CMJ, DJ and DDJ, NC for CON *CMJ 70.2 ± 5.6 to 75.6 ± 6.3 μg/L**** *DJ 71.0 ±5.5 to 77.6 ± 5.8 μg/L**** *DDJ 73.0 ± 6.3 to 80.8 ± 6.8 μg/L**** *CON 71.9 ± 5.3 to 70.1 ± 5.6 μg/L* tOC ↑ for DJ only NC for CMJ, DDJ and CON *CMJ 31.2 ± 2.3 to 32.2 ± 2.4 μg/L* *DJ 30.7 ±2.2 to 32.4 ± 2.5 μg/L*** *DDJ 30.6 ± 2.2 to 31.8 ± 2.3 μg/L* *CON 31.1 ± 2.1 to 30.0 ± 2.0 μg/L* NC for CTX all sessions *CMJ 636.0 ± 83.4 to 635.5 ± 80.3 ng/L* *DJ 645.2 ± 88.3 to 666.2 ± 91.0 ng/L*

Study details Author, journal (year)	**Study design**	**Participants** Sample (n, M/F) Age (years) Height (cm) Weight (kg) or BMI (kg/m^2) mean ± SD	**Acute exercise intervention** T: type D: duration I: intensity S&R: sets and reps	**Blood sampling protocol** T: timepoints C: controls B: BTMs measured	**Main findings** Effects of acute exercise compared to baseline
			Session 3- IMPACT T: 7 min low impact warm up on a gymnastic mat then DDJ (above) but performed diagonally forward 45° **For session 1 to 3:** D: not stated I: not stated S&R: 6 sets of 10 reps interspersed with 90 sec rest **Session 4 CON** T: complete rest		*DDJ 612.8 ± 85.9 to 632.8 ± 85.4 ng/L* *CON 590 ± 73.6 to 582.4 ± 74.4 ng/L* **Post exercise at 2 hrs** NC for P1NP all sessions *CMJ 70.2 ± 5.6 to 68.7 ± 6.0 µg/L* *DJ 71.0 ±5.5 to 67.5 ± 6.0 µg/L* *DDJ 73.0 ± 6.3 to 70.2 ± 6.0 µg/L* *CON 71.9 ± 5.3 to 70.6 ± 5.4 µg/L* tOC ↓ for CMJ, DJ and CON only *CMJ 31.2 ± 2.3 to 28.9 ± 2.2 µg/L*** *DJ 30.7 ±2.2 to 28.3 ± 2.5 µg/L*** *DDJ 30.6 ± 2.2 to 29.2 ± 2.2 µg/L* *CON 31.1 ± 2.1 to 28.1 ± 2.0 µg/L*** CTX ↓ for all sessions *CMJ 636.0 ± 83.4 to 527.9 ± 65.7 ng/L*** *DJ 645.2 ± 88.3 to 525.5 ±69.0 ng/L*** *DDJ 612.8 ± 85.9 to 519.0 ± 69.1 ng/L*** *CON 590 ± 73.6 to 501.7 ± 65.8 ng/L***

Study details Author, journal (year)	**Study design**	**Participants** Sample (n, M/F) Age (years) Height (cm) Weight (kg) or BMI (kg/m^2) mean ± SD	**Acute exercise intervention** T: type D: duration I: intensity S&R: sets and reps	**Blood sampling protocol** T: timepoints C: controls B: BTMs measured	**Main findings** Effects of acute exercise compared to baseline
Rudberg et al. Calcif Tissue Int (2000) (60)	Non-randomised, single arm	**Postmenopausal women** n= 8 57 ± 4 yrs 164 ± 5 cm 69.5 ± 9.6 kg 25.9 ± 3.6 kg/m^2	**1. AER** T: cycle GXT D: average duration of test was 24 mins I: workload ↑ by 30 W every 6 min (start 30 W) until exhaustion	T: baseline, post ex (+0 and 20 min) C: PM, 2hrs post feed (NFT) B: ICTP, ALP total, B-ALP 1 and 2 and tOC	**Post exercise at 0 min** (all mean ± SD) ↑ ALP total, ALP B/I, AP B1, ALP B2, ALP L1, ALP L3 NC all other markers *ALP total 3.08 ± 0.73 to 3.40 ± 0.70 ukat/L* ** *ALP B/I 0.12 ± 0.06 to 0.15 ± 0.07 ukat/L* ** *ALP B1 0.50 ± 0.18 to 0.63 ± 0.21 ukat/L* ** *ALP B2 1.18 ± 0.45 to 1.49 ± 0.45 ukat/L* ** *ALP B1/B2 0.43 ± 0.07 to 0.43 ± 0.08%* *tOC 3.2± 1.4 to 2.9 ± 1.0 μg/L* *ICTP 2.8 ± 0.9 to 2.7 ± 0.8 μg/L* **Post exercise at 20 min** NS all markers *ALP total 3.08 ± 0.73 to 3.32 ± 0.84 ukat/L* *ALP B/I 0.12 ± 0.06 to 0.14 ± 0.07 ukat/L* *ALP B1 0.50 ± 0.18 to 0.61 ± 0.20 ukat/L* *ALP B2 1.18 ± 0.45 to 1.46 ± 0.49 ukat/L* *ALP B1/B2 0.43 ± 0.07 to 0.43 ± 0.09%* *tOC 3.2± 1.4 to 3.5 ± 1.2 μg/L* *ICTP 2.8 ± 0.9 to 2.4 ± 0.5 μg/L*

Study details Author, journal (year)	**Study design**	**Participants** Sample (n, M/F) Age (years) Height (cm) Weight (kg) or BMI (kg/m^2) mean ± SD	**Acute exercise intervention** T: type D: duration I: intensity S&R: sets and reps	**Blood sampling protocol** T: timepoints C: controls B: BTMs measured	**Main findings** Effects of acute exercise compared to baseline
Kristoffersson et al. Eur J Exp Musculoskel Res (1995) (58)	Single arm	**Early postmenopausal women** n= 15 55 ± 3.87 yrs 165 ± 3.87 cm 65.0 ± 7.75 kg 23.7 ± 2.32 kg/m^2 1.06 ± 0.03 g/cm^2 Total BMD	**1. AER** T: jogging (6 degrees) D: 45mins I: 50% of VO^2_{Max} estimated by 50% of HR_{Max} reserve	T: baseline, post ex (+1, 24 and 72 hr) C: no-exercise for 3 days prior/post, AM, NFT B: P1CP, ICTP, and tOC	**Post exercise at 1 hr** (all mean ± SEM) ↑ tOC *tOC 4.8 ± 0.4 to 5.7 ± 0.5 μg/L* ** *PICP 129 ± 15 to 128 ± 15 μg/L* *ICTP 2.33 ± 0.25 to 2.48 ± 0.17μg/L* **Post exercise at 24 hr** NC tOC, PICP or ICTP *tOC 4.8 ± 0.4 to 5.5 ± 0.6 μg/L* *PICP 129 ± 15 to 134 ± 13 μg/L* *ICTP 2.33 ± 0.25 to 2.61 ± 0.21μg/L* **Post exercise at 72 hr** NC tOC, PICP or ICTP *tOC 4.8 ± 0.4 to 5.3 ± 0.5 μg/L* *PICP 129 ± 15 to 140 ± 12 μg/L* *ICTP 2.33 ± 0.25 to 2.61 ± 0.21μg/L*
Zerath et al. Med Sci Sp Exerc. (1997) (59)	Single arm	**Healthy active males** n= 24 62.3 ± 5.39 yrs 172.3 ± 5.39 cm	**1. AER** T: maximal cycle GXT D: ~ 10 mins I: increased by 20 W every 2 min until exhaustion	T: baseline, post ex (+0 to 1 min) C: AM, OFT B: ALP, tOC	**Post exercise at 0-1 min** (all mean ± SEM) ↑ ALP and tOC *ALP 41.7 ± 3.5 to 47.8 ± 3.7 μl/L^{-1}* * *tOC 6.18 ± 0.44 to 7.01 ± 0.36 ng.mL^{-1}* *

Study details Author, journal (year)	**Study design**	**Participants** Sample (n, M/F) Age (years) Height (cm) Weight (kg) or BMI (kg/m^2) mean ± SD	**Acute exercise intervention** T: type D: duration I: intensity S&R: sets and reps	**Blood sampling protocol** T: timepoints C: controls B: BTMs measured	**Main findings** Effects of acute exercise compared to baseline
		71.9 ± 8.33 kg			
		Older adults mean age >65 years			
Aly et al. Geriatric Med and Care (2017) (57)	Single arm	**# Elderly men & women** n= 40 (26/14) 66.2 ± 6.3 yrs 163.64 ± 26.44 cm 71 ± 5.5 kg 25.24 ± 2.15 kg/m^2	**1. AER** T: treadmill GXT D: ~10 mins I: 3 min warm up @ 40% age predicted HR_{Max}, gradual increase of exercise intensity until reaching 75 to 85% calculated HR_{Max}	T: baseline, post ex (+10 to 30 sec) C: NFT B: ALP	**Post exercise at 10-30 sec** (all mean ± SD) ↑ ALP *ALP 63.76 ± 19.24 to 75.4 ± 21.9* **
Kim et al J Exerc Nutr Biochem (2014) (56)	Single arm	**Elderly osteopenic women** n= 11 (5 osteoporotic) 68.18 ± 3.19 yrs 151.24 ± 2.94 cm 54.29 ± 5.21 kg 23.73 ± 2.07 kg/m^2 -2.51 ± 0.47 T-score	All participants completed in the same order (1 week apart) **1. CON** T: nil intervention, rest in chair D: not stated **2. RE** T: pilates exercises	T: baseline, post ex (+0 and 60 min) C: At least 12 hr of fasting B: ALP	**Post exercise at 0 min** (all mean ± SD) *CON 60.2 ± 13.3 to 60.2 14.0* *RE 59.**1** ± 14.0 to 58.5 ± 14.2* **Compared to baseline at 60 min** ↓ ALP at 60min *CON 60.2 ± 13.3 to 58.9 ± 13.6* *RE 59.1 ± 14.0 to 57.1 ± 13.8***

Study details Author, journal (year)	**Study design**	**Participants** Sample (n, M/F) Age (years) Height (cm) Weight (kg) or BMI (kg/m^2) mean ± SD	**Acute exercise intervention** T: type D: duration I: intensity S&R: sets and reps	**Blood sampling protocol** T: timepoints C: controls B: BTMs measured	**Main findings** Effects of acute exercise compared to baseline
		*T-score is whole body	D: ~70 mins including warm up and 50 mins of pilates exercises I: warm up (RPE 9 to 12), pilates exercises (RPE 10 to 14) S&R: not stated		
Maimoun et al. Br J Sp Med (2005) (52)	Non-randomised, single arm, group comparison	**Active elderly** n= 21 (11/10) 73.3 ± 9.1 yrs 166.3 ± 9.2 cm 65.8 ± 13.2 kg 23.6 ± 2.9 kg/m^2	**1. AER** T: maximal treadmill GXT at preferred walking speed including a warm up walking at 0% grade, followed by 1 to 2% gradient increase until exhaustion D: 5 min warm up followed by maximal incremental test of 8 to 12 mins duration I: maximal	T: baseline, post ex (+0 min) C: OFT, AM B: CTX, tOC and BALP	**2005 study post exercise at 0 min** (all mean ± SD) NC all markers *CTX 5998 ± 3045 to 5959 ± 2866 pmol l^{-1}* *tOC 12.7 ± 5.5 to 12.5 ± 5.3 ng ml^{-1}* *BALP 13.1 ± 4.8 to 13.2 ± 4.7 ng ml^{-1}*
follow up study: **Maimoun et al.** J Sci & Sp. Med (2009) (51)		*follow up study* n= 45 ***Active*** n= 18 (10/8) 71.7 ± 8.6 yrs 166.9 ± 9.3 cm			**2009 study post exercise at 0 min** ↑ BALP for moderately active group only, NC all other markers Active *CTX 5998 ± 3045 to 5959 ± 2866 pmol l^{-1}*

Study details Author, journal (year)	**Study design**	**Participants** Sample (n, M/F) Age (years) Height (cm) Weight (kg) or BMI (kg/m^2) mean ± SD	**Acute exercise intervention** T: type D: duration I: intensity S&R: sets and reps	**Blood sampling protocol** T: timepoints C: controls B: BTMs measured	**Main findings** Effects of acute exercise compared to baseline
		66.1 ± 13.3 kg 23.5 ± 2.9 kg/m^2 ***Moderately active*** n= 18 (10/8) 71.9 ± 7.3 yrs 166.6 ± 7.8 cm 70.7 ± 12.7 kg 25.3 ± 3.2 kg/m^2			*tOC 12.7 ± 5.5 to 12.5 ± 5.3 ng ml^{-1}* *BALP 13.1 ± 4.8 to 13.2 ± 4.7 ng ml^{-1}* Moderately active *CTX 5595 ± 2460 to 5385 ± 2201 pmol l^{-1}* *tOC 12.2 ± 4.5 to 12.6 ± 3.9 ng ml^{-1}* *BALP 11.6 ± 2.9 to 13.0 ± 4.1 ng ml^{-1}* *
Thorsen et al. Calcific Tissue Int (1996) (61)	Single arm	**Postmenopausal women** n= 12 68 ±3.46 yrs 167 ± 3.46 cm 71.2 ± 7.97 kg 25.3 ± 2.08 kg/m^2 1.05 ± 0.03 g/cm^2 total BMD	**1. AER** T: brisk walking (-2 degrees) D: 90 mins I: 50% of VO^2_{Max} estimated by 50% of HR_{Max} reserve	T: baseline, post ex (+1, 24 and 72 hr) C: AM, NFT, no-exercise for 3 days prior or post B: ICTP, tOC, P1CP	**Post exercise at 1 hr** (all mean ± SEM) NC tOC or PICP, ↓ in ICTP *tOC 7.3 ± 0.5 to 7.4 ± 0.4 μg/L* *PICP 139 ± 11 to 132 ±10 μg/L* *ICTP 2.88 ± 0.12 to 2.48 ± 0.19μg/L* * **Post exercise at 24 hr** NC tOC or ICTP, ↑ PICP *tOC 7.3 ± 0.5 to 6.9 ± 0.5 μg/L* *PICP 139 ± 11 to 155 ±13 μg/L* ** *ICTP 2.88 ± 0.12 to 3.18 ± 0.32 μg/L*

Study details Author, journal (year)	**Study design**	**Participants** Sample (n, M/F) Age (years) Height (cm) Weight (kg) or BMI (kg/m^2) mean ± SD	**Acute exercise intervention** T: type D: duration I: intensity S&R: sets and reps	**Blood sampling protocol** T: timepoints C: controls B: BTMs measured	**Main findings** Effects of acute exercise compared to baseline
					Post exercise at 72 hr NC tOC, ↑ PICP, ↑ in ICTP *tOC 7.3 ± 0.5 to 7.4 ± 0.8 μg/L* *PICP 139 ± 11 to 157 ±11 μg/L* ** *ICTP 2.88 ± 0.12 to 3.33 ± 0.21 μg/L* **
Wherry et al. Med & Sci Sports Ex. (2019) (47)	Non-randomised, uncontrolled crossover	**Healthy recreationally active older adults** n= 12 (5/7) 67 ± 5 yrs 1.7 ± 0.1 m 67.7 ± 15.9 kg -1.6 ± 0.6 T-score (T-score is femoral neck)	Two acute bouts of treadmill walking performed 1 to 4 weeks apart under cool and warm conditions **1. AER** T: treadmill walking D: 60 mins (+ 5min warm up and 5 min cool down) I: 70 to 80% of HR_{Max}	T: baseline, post ex (peak, +15, 30, 45 and 60 min) C: NFT, controlled pre-feed B: CTX	**Post exercise at peak** (all mean ± SD) ↑ CTX both conditions *Cool 0.255 ± 0.14 to 0.355 ± 0.17 ng/mL* * *Warm 0.255 ± 0.14 to 0.309 ± 0.114 ng/mL* * **Post exercise at 15 mins** ↑ CTX both conditions *Cool 0.255 ± 0.14 to 0.353 ± 0.163 ng/mL* * *Warm 0.255 ± 0.14 to 0.353 ± 0.163 ng/mL* * **Post exercise at 30 mins** ↑ CTX both conditions *Cool 0.255 ± 0.14 to 0.375 ± 0.16 ng/mL* * *Warm 0.255 ± 0.14 to 0.348 ± 0.115 ng/mL* * **Post exercise at 45 mins** ↑ CTX both conditions

Study details Author, journal (year)	**Study design**	**Participants** Sample (n, M/F) Age (years) Height (cm) Weight (kg) or BMI (kg/m^2) mean ± SD	**Acute exercise intervention** T: type D: duration I: intensity S&R: sets and reps	**Blood sampling protocol** T: timepoints C: controls B: BTMs measured	**Main findings** Effects of acute exercise compared to baseline
					*Cool 0.255 ± 0.14 to 0.364 ± 0.184 ng/mL **
					*Warm 0.255 ± 0.14 to 0.365 ± 0.127 ng/mL **
					Post exercise at 60 mins
					↑ CTX both conditions
					*Cool 0.255 ± 0.14 to 0.400 ± 0.177 ng/mL **
					*Warm 0.255 ± 0.14 to 0.391± 0.129 ng/mL **
					*changes not different between conditions

Keywords: RCT, randomised controlled trial; M, male; F, female; PoM, post-menopause; FT, fasting; OFT, overnight fasted; NFT, not fasted; AM, performed in morning; PM, performed in afternoon; RE, resistance exercise; RE+IMP, resistance and impact exercise; IMPACT, impact only exercise; 1RM, one repetition maximum) AER, aerobic exercise, CON, control; GXT, graded exercise test; ALP, alkaline phosphatase; BALP, bone specific alkaline phosphatase; PICP, Procollagen I Carboxyterminal Propeptide; P1NP, Procollagen Type 1 N Propeptide; OC, osteocalcin; ucOC, undercarboxylated osteocalcin; CTX, C-Terminal Crosslinked Telopeptide of Type I Collagen; ICTP, Cross-Linked Carboxyterminal Telopeptide of Type I Collagen; SCL, sclerostin

NC, no change compared to baseline or control; ↑, significant increase compared to baseline or control; ↓, significant decrease compared to baseline or control, *p= <0.05, **p= <0.01

5.4.3 Acute aerobic exercise

5.4.3.1 *Effects on BTMs: middle-aged adults*

Two studies reported significant increases in ALP immediately following cycling GXTs performed to exhaustion in men and in middle-aged postmenopausal women (59, 60). BALP also increased (range ~0.7 to 26%) in women after a cycling GXT to exertion, and also after moderate intensity walking (46mins, 3-6 METs) (54, 60). Three studies reported significant increase in tOC (range ~13.4 to 18.8%) in men who cycled (GXT to exertion; and 75% VO^2_{Peak}, 30mins), and in middle-aged postmenopausal women who jogged (50% HR_{Max} reserve, 45mins) (48, 58, 59). However, three cycling studies reported no change in tOC, one in men (90-95% HR_{Peak}, 30 mins) and two in middle-aged postmenopausal women (70-75% VO^2_{Peak}, 30mins; GXT to exertion) (49, 55, 60). No significant change was reported in P1NP after cycling in middle-aged postmenopausal women (70-75% VO^2_{Peak}, 30mins) (55) or in men (90-95% HR_{Peak}, 30mins) (49). Acute AE was also reported to have no effect on PICP in middle-aged postmenopausal women after jogging (50% HR_{Max} reserve, 45mins) (58).

One study reported that acute AE significantly increased (~16.6%) ß-CTX after cycling in men (90-95% HR_{Peak}, 30mins), however, there was no change in ß-CTX after cycling (75% VO^2_{Peak}, 30mins) or CTX after walking (3-6 METs, 46mins) in middle-aged postmenopausal women (49, 54, 55). Two studies measured ICTP with no significant changes in middle-aged postmenopausal women after jogging (50% HR_{Max} reserve, 45mins) or cycling (to exertion, GXT) (58, 60). SCL was reported to increase following brisk walking in middle-aged postmenopausal women (3-6 METs, 46mins) (54).

5.4.3.2 *Effects on BTMs: older adults*

ALP significantly increased in men and women immediately following a treadmill GXT (stopped at 75-85% HR_{Max}) (57). BALP also significantly increased (~12%) immediately following a treadmill GXT (to exertion), but only in men and women who were classed as moderately active (classified using a physical activity questionnaire) and not active based on baseline exercise levels (52). Two studies reported that tOC did not change in women after walking (50% HR_{Max} reserve, 90mins) or in men and women after

a treadmill GXT (to exertion) (52, 61). PICP was reported to increase in women after walking (50% HR_{Max} reserve, 90mins) (61).

Wherry et al. (47) reported significant increases (range 34.6 to 77.3 %) in CTX levels at all post-exercise time points (peak, 15, 30, 45 and 60mins) in men and women who walked at moderate intensity (70-80% $HR_{Max,}$ 60mins). In contrast, Maimoun et al (52) reported no significant change in men and women following a maximal GXT (treadmill). Thorsen et al (61) reported a significant decrease (~13.8%) in 1CTP levels at 1hr, but a significant increase (~15.5%) in levels at 72hrs post brisk walking (50% HR_{Max} reserve, 90mins).

5.4.4 Acute resistance with and without impact, or impact alone exercise

5.4.4.1 *Effects on BTMs: Middle-aged and older adults*

The effect of acute RE with and without impact exercises, versus impact only exercise on BTMs greatly varied with a limited number of studies measuring the same BTMs. Studies involving RE+impact, no change was reported in BALP in middle-aged postmenopausal women, or in tOC in middle-aged men (48, 54). On the contrary, impact-only exercise (three forms of jumping, *see Table 3*) significantly increased tOC (double jump group) and P1NP (all groups) immediately post, but at 2-hours tOC significantly decreased (all groups), with P1NP also reducing (non-significant) to below baseline levels (53). The drop in tOC (significant) and P1NP (non-significant) to below baseline levels was consistent with the control group in that study (53). CTX was the only consistent measured bone resorption marker shown to decrease following RE+impact and impact-alone protocols in middle-aged women (53, 54). However, in the impact-alone study, the significant decrease at 2-hours post (not immediately after) was not significantly different to the control group (53). Only one study investigated acute RE in older women (56) and reported a significant decrease in ALP; no other BTMs were measured in this study.

5.5 Discussion

We report that a) BTM responses to acute exercise vary between middle- and older-aged adults and that the BTM responses may be b) sex-specific and c) altered by exercise mode, intensity and duration. Additionally, responses to acute exercise stimuli may be d) BTM-specific, with some markers being more sensitive than others to the same

stimuli. We identified a major gap in the current field with a small number of studies investigating acute effects of exercise on BTMs in middle-aged adults (n= 8), and even fewer number in older-adults (n= 5).

The application of mechanical stress (i.e. exercise) to the skeleton can preserve and increase BMD, serving as a key intervention in the prevention and management of osteoporosis (8-10). The effect of chronic, long-term, exercise on BMD in older adults is well established, shown to be modality- (AE, RE and impact-loading) and intensity-dependent (9, 39, 62, 63). Evidence suggests that even walking is of limited value for improving bone health if not prescribed with features that increase loading (36, 39, 62, 64-66). It is well accepted that RE with weight bearing and high impact is safe and effective to optimise bone health in older adults, as they result in high strain rates and peak forces and, reduce falls and fractures (7, 9, 35, 37, 67). In fact, high-velocity power and rapid concentric contractions (inducing higher strain rates on bone) is more beneficial for functional performance (i.e. chair rise) in older adults (68-70). Additionally, regular weight-bearing impact, applied in multidirectional patterns, promotes bone maintenance/preservation (62, 71). While the evidence is clear from chronic, long term, exercise training studies what characteristics exercise protocols should consist of for beneficial effects on bone health in adults, the effects of acute exercise are unclear. Available data are conflicting and, as it is not appropriate to measure BMD after a single session, BTMs are used as a surrogate measure (41). Whether various modes of acute exercise with different modifiable characteristics alter bone metabolism differently in middle and older adults is underexplored.

5.5.1 Age and sex-specific effects on BTM responses to acute exercise

Based on this review, while acute exercise is sufficient to detect responses in BTMs, these responses may be age- and sex-specific, highlighting some possible consideration in the design of future acute exercise studies. For instance, all AE exercise studies investigating the tOC and BALP response in older adults (men and women) report no change after exercise, but some studies in middle-aged adults (men and women) report increases (48, 52, 54, 58-60). Conversely, ALP appears to have similar sensitivity in middle and older aged men and women (49, 57, 59, 60) and resorption markers CTX (men and women) and ICTP (women only) appear to increase in older adults, but not middle-aged (47, 54, 58, 60, 61). Lastly, tOC and ß-CTX responses to AE also appears to be more

sensitive in middle-aged men than women, suggesting a possible sex-specific response (48, 55, 58-60). Differences in BTM responses between middle- and older-aged adults could be multifactorial, explained by age-related alterations to bone composition and hence bone turnover, and in women, menopausal effects, possibly altering the bone response (6, 72-76). Indeed, underlying bone pathophysiology is different in middle-aged vs older women who, are known to have elevated bone turnover rates, possibly explaining differences in responses (6, 77). Given bone resorption was not significantly altered in some of these studies in women (54, 55, 58, 60) may in fact, be beneficial (not stimulating further the negative balance of the remodelling process), however this is poorly understood and warrants further exploration.

Of note, at baseline, some studies did not report/screen for bone health indices, as adults are known to be affected by age-related bone composition alterations, particularly women, this should be considered. Some studies excluded individuals with osteoporosis (48, 49, 52, 53), whereas others included adults with osteopenia/osteoporosis (47, 54, 56), possibly influencing BTM responses (78). Some studies in older adults pooled men and women data together (47, 57), only one confirming no sex-interaction in BTM responses (52). As older women are known to have different rates of bone turnover and consequently accelerated bone loss compared to men, bone responses may be altered (or attenuated) thus, men and women should be handled separately, or sensitivity tests performed (34, 72-76, 78).

5.5.2 BTM responses modulated by exercise mode, intensity, and duration

This review summarises that BTM responses to acute exercise may be modulated by the specific characteristics of the exercise protocol used. For instance, a majority of studies report no change in tOC following AE regardless of intensity (low, moderate, high) (49, 52, 55, 60, 61). However, tOC may be more sensitive only to AE that incorporates loads of greater ground-reaction force increasing in one study after jogging, but not after the majority of studies including cycling or walking protocols (49, 52, 55, 58, 60, 61). Whereas, ALP, BALP and PICP increase after cycling and walking, suggesting these markers have higher sensitivity to AE with lower impact (52, 54, 57, 59-61). Indeed, in three separate studies in middle-aged men utilising cycling protocols the tOC response was different, increasing only after moderate intensity cycling (30mins) and a short duration maximal exertion GXT, but not after high-intensity interval exercise

(30mins) (48, 49, 59). This suggests that exercise intensity and duration may be important, but there may be other possible modulating effects on the tOC response, which should be further explored. Markers reflecting bone resorption, CTX and ICTP appear to be more sensitive to AE protocols that are longer (≥ 60mins), not shorter duration (<45mins) (47, 52, 54, 58, 60, 61). Whereas, ß-CTX (a different fragment of CTX) responds differently to cycling exercise of same duration (30mins), increasing only after high-intensity, but not moderate-intensity cycling, suggesting that in this instance, intensity may be important (49, 55).

Despite the mounting evidence for the use of RE combined with weightbearing and impact loads distributed in dynamic and novel patterns for optimising bone health effects, little is known about the acute effects and available studies investigating these characteristics is limited. Based on this review, RE with impact does not stimulate a response in markers reflecting bone formation (48, 54). However, one study measured BALP only at immediately post exercise (54), the other measured tOC only up to 2-hours, possibly missing the kinetic response (48). Direct comparison of these study protocols is difficult, one study used core stabilisation bodyweight exercises with small impact exercises (steps, hopping) (54), the other study used power leg press RE (70 to 75% maximal strength) with high impact jumping, thus the impact and mechanical strain load on bone would be very different (48). However, it does appear that high impact exercise alone and RE alone is sufficient to detect a response in BTMs of formation. Indeed, ALP was decreased in one study following a RE regimen of pilates exercises, however, whether this is truly indicative of a bone-response is unclear, and other BTMs were not measured (41, 56, 79). Of note, only the study investigating impact alone using three sessions each containing a different form of jumping, reported increases of tOC and P1NP. P1NP increased for all jumping protocols, but tOC was only increased in the session where participants dropped from a height to an explosive vertical jump, not from jumping directly from the floor (53). Highlighting that, P1NP may be more sensitive than tOC to impact exercise, and that the tOC-specific response may require greater impact loads (ground reaction force) combined with high explosive movements to elicit a response. Based on these studies it appears that CTX decreases with RE combined with impact, and with impact alone protocols (53, 54). However, while both of these studies were RCTs, the impact only study which was crossover in design report that CTX decreases also in the control condition (53). This decrease was not different to the

decrease seen post the impact exercise, indicating that CTX is affected by circadian/diurnal effects (53, 80).

Altogether, the evidence from this review, and from the literature demonstrates that exercise intensity, dynamic, and novelty of new loads (non-habitual nature) placed on the skeleton are important characteristics influencing the bone-exercise response (16, 18, 23, 81, 82). However, only three studies included participants' baseline fitness in the selection criteria (47, 48, 53). Three state (58, 60, 61) participants were non-regular exercisers, but one reports participants regularly cycling (1-6km/day, few days a week) (60). As habitual exercise was not considered in a majority of studies, protocols may lack in specificity, and although some used prior testing to define exercise intensity their protocols possibly lack in novelty of new load (12, 23, 83). Indeed, one interesting concept, explored by one study, was the possible effect on the BTM response based on the participants baseline fitness, whereby BALP was only shown to be significantly increased with AE exercise when older adults were further stratified into moderately active, or active groups (52). This possibly suggests that the BALP response in older adults may be dampened, modulated by the participants' baseline fitness, supporting the principle that bone cells have a threshold level of adaptation and the need for consideration of individualised, progressive (graded, based on baseline fitness) and novelty in protocol loads, discussed earlier (12, 23, 83). This should be further explored in future research, as it likely impacts/dampens the BTM-response and therefore a skewness in results.

5.5.3 BTM-specific responses to acute exercise

To understand if different BTMs thought to reflect the same bone turnover phase have different sensitivities to acute exercise we compared study effects where >1 BTM reflecting the same bone formation or resorption phase was measured within the same study. AE appears to have a limited effect on tOC and P1NP, whereas other markers reflecting bone formation namely ALP, BALP and PICP appear to be more sensitive. Altogether, suggesting that tOC may be the least sensitive BTM of formation and supports the notion that these BTMs may represent different phases of osteoblastic function or formation (41). Indeed, ALP activity includes serum derived from liver and bone, therefore changes in response of ALP may be non-specific to bone, as such BALP is recommended for its increased specificity (41, 79).

While AE appears to have a limited effect on tOC, one concept to raise about tOC is that it exists in the circulation in a carboxylated (cOC) reflecting more bone mineralisation, and undercarboxylated (ucOC) form, considered the more "bio-active" counterpart, acting as a hormone involved in energy metabolism and possibly a role in muscle maintenance and strength (84-90). When studies measured effects on tOC only, whether there is a shift in favor of cOC, or ucOC, is unclear, as only few studies measured this (48, 49, 55). In these studies, ucOC increased even with null change in tOC in two of them (49, 55). Therefore, regarding tOC, there is much more to be understood.

One study measured >1 BTM reflecting resorption, interestingly SCL, a possible promoter of bone resorption, increased following walking, but not CTX (54, 91). Suggesting, SCL may be more sensitive than CTX, however, blood sampling was performed only once (immediately post) possibly missing peak change in CTX. Of note, SCL increases with age and high levels are associated with long-term physical in-activity/immobilisation (92-95). Additionally, mechanical unloading increases the expression (gene and protein) of SCL, whereas SCL expression decreases with mechanical loading (*in-vivo* and *in-vitro*) (96, 97). Therefore, SCL may be an interesting marker to be included in future studies.

BTMs are highly dynamic and sensitive, however, investigators should consider factors known to influence BTMs in preparation for testing i.e. circadian/diurnal rhythm, feeding, sleep, smoking, menopause age and exercise (41, 42, 74-76). Some studies were not performed in the fasted state and/or in the morning (47, 57, 58, 60, 61). In addition, blood sampling protocols largely differed between included studies, some sampling only immediately post exercise, others taking multiple samples but up to 2-hours post exercise, and others up to 72-hours post. As blood sampling represents only a small "snapshot window in time" it is possible, that at least those that only sampled immediately post may have missed the peak response of the BTM-kinetics. While there are some ethical considerations for invasive techniques and frequency of venepuncture and/or sampling volume, a better understanding of the time-course response of BTM-kinetics is required. Despite advances in quality assurance, laboratory errors commonly occur in pre-analytical phases i.e. timing of sampling, selection of specimen, collection procedure and, sample transport, temperature and time to storage, thus extra rigor should be employed to ensure accurate and reproducible results (42, 43, 98, 99).

5.5.4 Limitations and strengths

To our knowledge this is the first systematic review to examine effects of acute exercise on BTMs in adults >50 years of age, highlighting major gaps in the field and considerations for increased rigor in future trials. The current review emphasises that research into the effects of acute exercise on BTMs in middle-aged adults is limited and is even scarcer in older adults. Whilst the number of included studies is low (n = 13), it covers the only available research in this area. Several factors limit the generalisability of the findings; lack of RCTs, low quality of the evidence, small sample sizes, potential bias in the cohorts, large variance in the exercise and blood sampling protocols, and the use of different assays to detect BTMs.

5.5.5 Conclusions

Acute exercise is an effective tool to induce changes in serum BTMs, however, the response appears to be exercise modality-, intensity-, age- and sex-specific. Large variability in study populations, exercise and blood sampling protocols explains conflicting results and as such, future studies should include tight control over factors that influence BTMs. Longer sampling periods of BTMs may assist in understanding the BTMs-kinetic responses. As is, the understanding of the influence of the possible endocrine regulation related to these BTMs. Further high-quality acute exercise studies are needed to identify new mechanistic target pathways for therapeutics and optimising exercise prescription for adults.

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5.7 Supplementary materials

Supplementary 1 PICOS protocol

Criteria for study inclusion

Participants

- Community dwelling, middle aged, and older adults > 50 years of age

Intervention

- Acute, single bout exercise including i.e. aerobic, resistance or other strength or fitness regime including jumping or circuit exercise.

Outcome Measures

Bone turnover markers including;

- Total alkaline phosphatase ALP
- Bone alkaline phosphatase B-ALP
- Osteocalcin OC or undercarboxylated osteocalcin ucOC
- C-terminal propeptide of type 1 procollagen P1CP
- N-terminal propetide of type 1 pro-collagen P1NP
- Cross-linked telopeptides of type I collagen include C terminal (CTX, carboxy-terminal cross-linked telopeptide) and N-terminal (NTX, amino-terminal cross-linked telopeptide).
- Sclerostin SCL

Study Design

- Randomised controlled trials
- Controlled trials including quasi-randomised
- Cross-sectional studies
- single arm studies

Supplementary 2 Literature search strategy

We developed search strategies to identify controlled trials involving acute exercise interventions in community dwelling, middle-aged and older adults >50 years of age. We focused on the effect of exercise on commonly used blood bio-markers of bone turnover (*see table 1*). We searched PubMed, SCOPUS, EMBASE and the Web of Science for studies published up to 22nd April 2020. Our systematic search strategy included the following terms; [adult OR elderly OR older adult OR post-menopausal OR middle aged OR older men OR older women] AND [exercise OR acute exercise OR single bout exercise OR aerobic exercise OR aerobic training OR resistance exercise OR resistance training OR circuit weight training OR jumping OR plyometric OR cycle OR cycling OR running OR vibration OR physical training or circuit training OR weight training OR high intensity iteral training OR weight lifting OR impact exercise OR impact training] AND [bone formation marker OR bone remodelling marker OR bone resorption marker OR bone turnover marker OR bone marker OR bone biomarker OR osteocalcin OR total alkaline phosphatase OR total alkaline phosphatase activity OR bone alkaline phosphatase OR propeptide OR type 1 pro-collagen OR type 1 collagen OR carboxy-terminal OR cross-linked telopeptide OR sclerostin].

Chapter 6: Higher bone remodelling biomarkers are related to a higher muscle function in older adults: effects of acute exercise

Context:

This study involved a randomised crossover clinical trial, including older adults >60 years of age. Based on previous studies, we utilised acute exercise as a novel tool to investigate bone-muscle cross-talk via, the measurement of bone biomarkers. Here, the trial included two modes of exercise, as it was identified that there was limited studies using this exercise mode, and we hypothesised, based on the known optimal loading characteristics for bone responses, that resistance exercise may have a greater response on bone that aerobic exercise. To further explore this bone-muscle relationship, this clinical trial included rigorous musculoskeletal health assessments (detailed in our published study protocol, appendices 1) and we performed cross sectional analyses on this relationship with bone biomarkers. The overarching conclusion of this study provided evidence that this link between muscle function and bone biomarkers, may not be limited to ucOC but BTMs in general. We also demonstrate that it is likely that the speeding of BTM responses to acute exercise are not likely representing underlying bone turnover, but probably a result of metabolic factors, explained in detail in this publication and in the general discussion.

The following paper, "Higher bone remodelling biomarkers are related to a higher muscle function in older adults: effects of acute exercise" is under review with Bone. This study was also presented at the following conferences:

- Australian and New Zealand Bone and Mineral Society, Poster and e'poster presentation (2020)

I also published a protocol paper for this larger study below (**Appendix 5**)

Smith, C., Lin, X., Scott, D., Brennan-Speranza, T. C., Al Saedi, A., Moreno-Asso, A., Woessner, M., Bani Hassan, E., Eynon, N., Duque, G., & Levinger, I. (2021). Uncovering the Bone-Muscle Interaction and Its Implications for the Health and Function of Older Adults (the Wellderly Project): Protocol for a Randomized Controlled Crossover Trial. *JMIR research protocols*, *10*(4),e18777. https://doi.org/10.2196/18777

Higher bone remodelling biomarkers are related to a higher muscle function in older adults: effects of acute exercise

Running title: Muscle function and bone biomarkers

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6.1 Abstract

It was suggested that the skeleton, via undercarboxylated osteocalcin (ucOC) might be linked to muscle mass and strength maintenance. However this relationship remains unclear and it may not be unique to ucOC, but to bone turnover markers (BTMS) in general. We tested the hypothesis that serum ucOC and bone turnover biomarkers are associated with muscle function, and that acute exercise could alter these serum levels. Thirty-five older adults (25 females/10 males, 72±6 years) participated. Baseline assessments included body composition (DXA), handgrip strength and a physical performance test (PPT) (gait speed, timed-up-and-go [TUG], stair ascent/descent). Leg muscle quality (LMQ) and stair climb power (SCP) were calculated. Participants performed (randomized) 30 mins aerobic (AE) (cycling 70%HR_{Peak}) and resistance (RE) (leg press 70%RM, jumping) exercise. C-terminal telopeptide of type I collagen (CTX), procollagen of type I propeptide (P1NP), total osteocalcin (t)OC and ucOC were assessed at baseline and post-exercise. Data were analyzed using linear mixed models and simple regressions, adjusted for sex. At baseline, higher muscle strength (LMQ, handgrip) was related to higher P1NP, higher SCP related to higher P1NP and ß-CTX, and better physical performance (lower PPT) related to higher P1NP and ß-CTX (p<.05). Exercise, regardless of mode, decreased ß-CTX and tOC (all p<.05), P1NP and ucOC were not altered. Post-exercise, lower ß-CTX was associated with higher baseline handgrip strength, SCP and LMQ. Poorer baseline mobility (increased TUG time) was associated with higher ß-CTX. Independently of exercise mode, acute exercise decreases ß-CTX and tOC. Our data suggests that in older adults the relationship between muscle quality/function and BTMs is not specific to ucOC, but BTMs in general. Furthermore, increased BTM levels was linked to better muscle function. Altogether, our data strengthens the evidence for bone-muscle interaction, however, mechanisms behind this specific component of bone-muscle cross-talk remain unclear.

Keywords: Aging; biochemical markers of bone turnover; exercise; bone-muscle interactions; skeletal muscle.

6.2 Introduction

In the last decade, there has been accumulating evidence that osteocalcin (OC) in its undercarboxylated form (ucOC) plays a role in bone-muscle cross-talk (1). Majority of mechanistic studies suggested that ucOC may be involved in muscle maintenance (mass and function) in rodents and *in vitro* studies (2-5). However, these findings was not supported by a recent study (6). In humans, there is some evidence demonstrating that ucOC and total (tOC) are related to muscle mass and strength (7-10), but data are limited to observational studies and often contradictory. For instance, in older females, higher ucOC/tOC was related to higher muscle strength in one study (7), while others reported higher ucOC/tOC was related to poorer physical function (timed-up-and-go, TUG) and a higher risk for falls-related hospitalization (8). In post-menopausal females who have previously had a fracture, lower ucOC was related to lower leg lean mass and higher falls risk (9). Additionally, adults with hypoparathyroidism treated with parathyroid hormone had increased ucOC/tOC, which was associated with increased elbow extension force (10). Importantly, it was elucidated that the link between bone and muscle is not specific for ucOC or tOC, but rather was suggestive of a broader link between bone remodelling (measured by serum biomarkers) and muscle (11, 12). Therefore, it is possible that other bone turnover markers (BTMs) used clinically to predict fracture risk, such as C-terminal telopeptide of type I collagen (CTX) and procollagen of type I propeptide (P1NP), are also involved in this specific aspect of the bone and muscle relationship (13).

Exercise in older adults is a non-pharmacological intervention known to improve muscle and bone health (14-18). Acute exercise can modify BTMs, but the effect is likely to be influenced by exercise mode and intensity, as well as by sex and age (19). Bone and muscle are closely linked anatomically and metabolically, and also share certain endocrine actions (1, 20). Indeed, both muscle and bone are regulated by mechanical loads, and evidence suggests bone mass may be tightly linked to skeletal muscle-derived mechanical loading (21-23). As such, the underlying muscle physiology (high or low mass/function) may partially explain bone biomarker responses to altered mechanical load, i.e. exercise (23-25). Altogether, mechanical load stimulated through acute exercise can be used as a tool to examine responses of bone biomarkers, and to explore whether these responses may be associated with muscle function and energy metabolism.

The aims of the current study were to: (a) perform cross-sectional analyses to determine the relationship of baseline determinants of muscle mass and function with

tOC, ucOC, CTX and P1NP and b) determine the effect of acute aerobic (AE) and resistance (RE) exercise on bone biomarker responses. A secondary aim of this study is to explore if baseline muscle mass and function are related to the bone biomarker responses following acute exercise.

6.3 Methods

6.3.1 Screening

The full protocol of this randomized crossover trial has been previously published, see **Figure 6.1** for study design schematic (26). Older adults >60 years without diabetes and not taking glucocorticoids were recruited from the community. Females were required to be a minimum of 12 months post-menopause. Exclusion criteria for participation included the presence of diabetes or taking any hyperglycaemic medications, had blood disorders, bone malignancies, taking warfarin or vitamin K supplementation or restriction, had a body mass index ≥40 kg/m, in the last 3 months reported any fractures or began new osteoporotic or antiresorptive treatments and engagement in a resistance exercise regimen for >2 sessions per week. Initially, 190 older adults were screened for eligibility, 118 did not meet the study criteria, or, following assessment by the study physician, were excluded due to complex medical status affecting their safe participation or primary study outcomes. Seventy-two were eligible, but 37 declined to participate, and 5 were not cleared to participate by their local medical practitioner. Thirty-eight participants were enrolled, 3 participants withdrew prior to study completion due to a change in medical status or medications. Thirty-five older adults (25 females, 10 males) completed the randomized, cross-over study. Participant medication or supplementation use included antihypertensives (n=16), cholesterol lowering (n=12), other heart medications or blood thinners (n=9), vitamin D (n=7), antidepressants (n=5), none were taking glucocorticoids. Each participant was given written and verbal explanation of the study prior to signing the consent form.

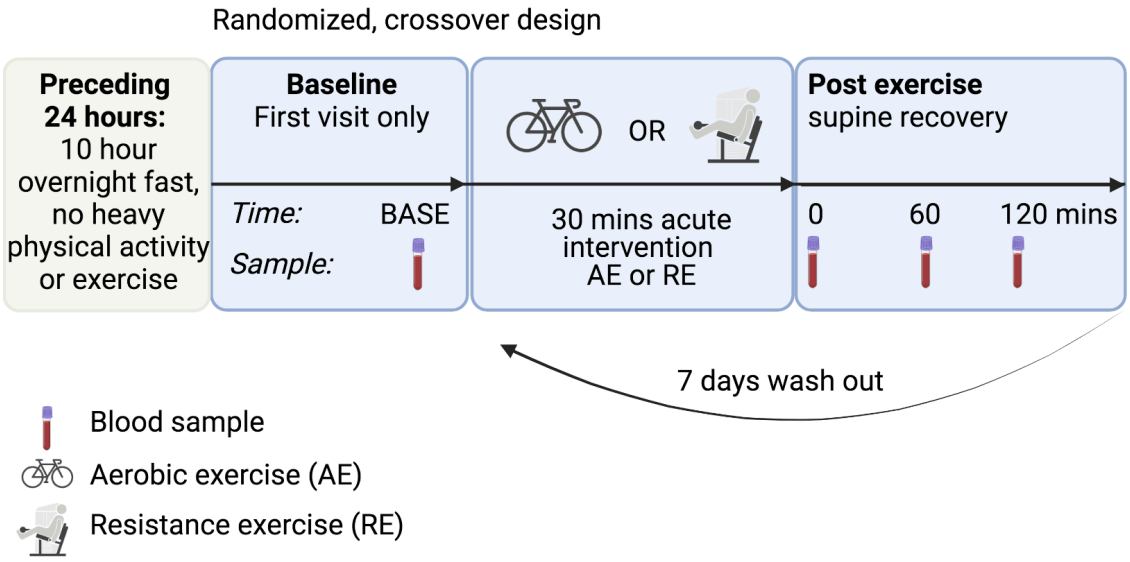

Figure 6.1 Study design. Figure created with BioRender.com

Baseline assessments occurred over two visits performed in the morning, following an overnight fast. Participants attended the laboratory where medical histories, current medications and anthropometric measurements were obtained, including body weight (digital scales) and height (stadiometer), and body mass index (BMI) was calculated (kg/m^2). Whole-body composition and bone mineral density (BMD) were measured via whole-body dual-energy X-ray absorptiometry (DXA, Hologic, Horizon A, software version 5.6.0.4).

Participants completed a graded exercise test (GXT) on a cycle ergometer to assess peak oxygen consumption ($\dot{V}O_{2Peak}$) and peak heart rate (HR_{Peak}). The GXT protocol started at 10-30 W increasing by 10 to 30 $W \times min^{-1}$ according to participant ability. Participants were monitored by 12-lead electrocardiogram (ECG; Mortara, X-Scribe II) and $\dot{V}O_2$ for each 15 sec interval by gas exchange analysis (Breeze, version 3.02, Medical Graphics Corp.). Tests were terminated according to participants' self-reported fatigue perception reaching a predetermined level (Borg scale Rating of Perceived Exertion=17) or clinical signs or symptoms [60]. Blood pressure was monitored using a manual sphygmomanometer and heart rate via the 12-lead ECG. HR_{Peak} obtained during the GXT was used to calculate the workload for the acute aerobic exercise (AE) session.

Handgrip strength was measured using a hand dynamometer, and gait velocity was measured using the instrumented walkway GAIT Rite system (CIR Systems Inc., Havertown, PA). Participants also performed a short physical performance test (PPT)

which involved four different mobility tasks: timed assessed via GAIT rite system, the TUG and a timed stair ascent and descent, which consisted of a rapid ascent and safe descent of 10 stairs. Briefly, the TUG is performed as time to rise from a chair (46cm), walk 3 m, turn, walk back to the chair and sit. All measures were completed three times, the best time was recorded. A PPT score was then calculated as the sum of fastest time for each task. In addition, using the time obtained for the stair ascent, stair climb power (SCP) was then calculated (power = force x velocity) (27). Velocity was calculated as the vertical distance of the stairs divided by the time to ascend, and force as the participant's body weight multiplied by acceleration due to gravity (9.8 m/s).

Leg muscle quality (LMQ), an estimate of specific force of a muscle group per unit of muscle mass, was calculated as follows: LMQ (kg/kg) = leg strength [kg[/(left leg lean mass [kg] + right leg lean mass [kg]) (28, 29). Maximal leg strength was measured on a leg press using the one-repetition maximum (1RM) test (30). The 1RM test was performed four to seven days after a familiarisation session and was used to calculate the intensity of the acute resistance exercise (RE) session.

6.3.2 Acute exercise intervention

Following the baseline assessments, participants completed two experimental sessions performed in a randomized order (sealed envelope method by an independent person to the study): one included acute aerobic (AE), and one acute resistance (RE) exercise conditions. Visits were ~ hours in duration, including the 30 min intervention (AE or RE) and were performed in the morning following an overnight fast. Twenty-four hours prior to their first session, participants completed a food diary, and this was replicated before the subsequent session. Participants were asked to refrain from structured exercise (48 hrs), limit physical activity (i.e. strenuous household chores) and alcohol ingestion (24 hrs) prior to testing visits. Visits were performed seven days apart, accounting for washout.

A resting, baseline venous blood sample was taken once (BASE) prior to the first session, and used as baseline for all visits, then the single session of 30mins of AE or RE was performed. AE included 30 mins of cycling at a moderate intensity (70 to 75% of HR_{Peak}), with the workload adjusted accordingly to achieve target HR. The target HR was calculated using the Karvonen heart rate reserve method: exercise target HR = (% of desired exercise intensity x (HR_{Peak}-HR_{rest}))+HR_{rest}. RE included 30 min of strength and

power exercises performed as leg press for 5 sets of 10 rapidly concentric (as fast as possible) and slow eccentric (4sec) repetitions at 70 to 75% of 1RM as well as jumping for 5 sets of 10 jumps (jumping as high as they can, safely, without stopping). Recovery between sets was 2 mins. Following the acute exercise intervention, three venous blood samples were taken, including immediately post-exercise (0 mins), as well as 60 mins and 120 mins thereafter. All participants recovered on a bed during the post-exercise recovery period. Procedures for the two sessions were identical.

6.3.3 Blood sampling and biochemical analysis

Venous blood was collected from an antecubital vein via an intravenous cannula with collection into ethylenediaminetetraacetic acid (EDTA) or clot activator serum separator tubes (SST). Blood samples were separated into plasma or serum via centrifugation (10min, at 3500rpm, 4°C). For serum only, centrifugation was completed following 10 mins of clotting time. Plasma/serum was subsequently aliquoted and stored at -80°C until analyzed. Serum ß-isomerized C-terminal telopeptides (ß-CTX) and procollagen 1 N-terminal propeptide (P1NP) were analyzed at the Medical University of Graz, Clinical Institute of Medical and Chemical Laboratory Diagnostics (Graz, Austria) and were measured using an electrochemiluminescence immunoassay (ECLIA) using a Cobas e immunoassay analyzer. Total serum osteocalcin (tOC) was measured using an automated immunoassay (Elecys 170; Roche Diagnostics, Mannheim, Germany). Serum ucOC was measured following Gundberg et al. (32) method by the same immunoassay after absorption of carboxylated OC on 5mg/mL hydroxyl-apatite slurry. Each sample was measured once and the inter-assay coefficients of variation were 5.4% and 9.2% for tOC and ucOC, respectively. Serum ucOC for 1 participant at timepoint 0 mins following AE was found to be a statistically significant outlier (>3SD away from the mean), and thus was excluded from the relevant analyses.

Fasting glucose levels in serum were analysed using an automated analysis system (YSI 2300 STAT Plus® Glucose & Lactate Analyzer). Fasting insulin levels in serum were measured via ELISA kit purchased from ALPCO, based on the manufacturer's instructions. Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was calculated using the formula: HOMA-IR = Glucose(mM) x Insulin (μU/mL) ÷ 22.5.

6.3.4 Study approval

The study was approved by and conducted in accordance with the Melbourne Health (MH) and Victoria University (VU) Human Research Ethics Committee's (HREC) (MHHREC: 2017/08) and was registered with the Australian New Zealand Clinical Trials Registry (trial number: ACTRN12618001756213).

6.3.5 Statistical methods

All statistical analyses were performed using R version 4.1.0. Linear mixed-effects models were used to examine a) if acute exercise alone (irrespective of exercise mode) or b) specific to exercise mode (AE and RE) could alter the levels of the biochemical variables of interest. Before running the linear mixed models, we calculated the Akaike information criterion (AICc), that corrects for small sample size to determine the most parsimonious model. All analyses were performed using a model where outcome was the bone biomarkers (ß-CTX, P1NP, tOC, ucOC); the fixed effects were group (AE or RE) and timepoint (BASE, and post-exercise blood sampling (0mins, 60mins and 120mins), the interaction between group (AE, and RE) and timepoint and finally we adjusted for sex. The random effect was the participants' unique ID, accounting for repeated measures. Using Spearman rho' correlations we examined whether changes in metabolic markers are associated with changes in BRMs post exercise. First, we calculated the percent change from baseline for 60mins and 120mins post exercise, which is what we have demonstrated previously to coincide with the peak change (31, 32), we then averaged these two together to get the average peak change post exercise. Simple linear regression were run to investigate whether outcomes related to muscle function explained any of the variability of bone biomarkers at baseline, and were adjusted for sex. PPT score for 1 participant was found to be a statistically significant outlier (5 SD away from the mean) and was excluded from the analyses. To meet the statistical assumptions of the regression (normally distributed residuals), P1NP, tOC and ucOC were log transformed. P values from the statistical analyses were adjusted for multiple testing using the false discovery rate (FDR) (33).The following packages were used in our analyses using R: lmerTest (34), tidyverse (35), lme4 (36), emmeans (37), MuMIn (38), sjstats (39), sjPlot (40), pbkrtest.

6.4 Results

Thirty-five older adults (10 Males, 25 Females) with mean age 72.8 ± 6.0 yrs and BMI of 28.3 ± 3.6 kg/m^2 were included in this study (**Table 6.1**).

Table 6.1 Baseline descriptive statistics

	All (n=35)
Sex (M/F)	10/25
Age (years)	72.83 ± 6.00
BMI (kg/m^2)	28.28 ± 3.59
Grip strength (kg)	30.63 ± 9.04
Gait velocity (m/s)	2.74 ± 0.53
SCP (W)	227.22 ± 77.30
PPT (s)	23.45 ± 8.63
Timed up and go (s)	8.61 ± 2.04
Leg muscle quality (kg/kg)	7.78 ± 3.02
VO_{2Peak} (ml.kg.min)	17.90 ± 4.04
Whole body BMD	1.07 ± .13
Whole body T-score	-.80 ± 1.47
Appendicular lean mass	6.74 ± 1.09
Biochemical measures	
ß-CTX (ng/mL)	0.44 ± 0.23
P1NP (ng/mL)	40.30 (34.55–56.75)
tOC (ng/mL)	21.68 (16.88-26.63)
ucOC (ng/mL)	8.18 (6.06-11.44)
ucOC/tOC (%)	40.45 ± 9.27
Insulin (pmol/L)	6.77 (5.05-8.41)
Glucose (mmol/L)	5.93±.65
HOMA-IR	1.76 (1.24-2.23)

BMD, bone mineral density; BMI, body mass index; SCP, stair climb power; PPT: physical performance test; ß-CTX, C-terminal telopeptide of type I collagen; P1NP, procollagen of type I propeptide; tOC, total osteocalcin; ucOC, undercarboxylated osteocalcin. Data reported as mean ± SD or median (IQRs).

6.4.1 Effects of acute exercise on bone biomarkers

Individual responses to acute exercise are shown in **Figure 6.2**. Regardless of mode of exercise, ß-CTX decreased by 0.02 ng/mL immediately post (0 mins), this

decrease did not remain significant after adjustment for multiple comparisons (β= -0.02 ng/mL, SE= 0.009, p= 0.03, FDR= 0.09) and by 0.03 ng/mL at 120 mins post-exercise (β= -0.03 ng/mL, SE= 0.009, p= 0.005, FDR= 0.04), compared to baseline. tOC was decreased by 6.5% at 120 mins post exercise compared to baseline, irrespective of mode of exercise (β= -0.063 log(ng/mL), SE= 0.01, p**< 0.001, FDR< 0.001)** (**Table 6.2).** There was no significant interaction between mode of exercise and time indicating that the response of ß-CTX and tOC to acute AE and RE were similar (**Supplementary Table 1).** P1NP, ucOC, ucOC/tOC and glucose were not significantly altered by either AE or RE. All analyses were adjusted for sex. The percent change in tOC and ucOC to acute exercise was inversely associated to the percent change in glucose post exercise (ρ= -0.44, p< 0.001; ρ= -0.31, p= 0.01 respectively) see **Figure 6.3**.

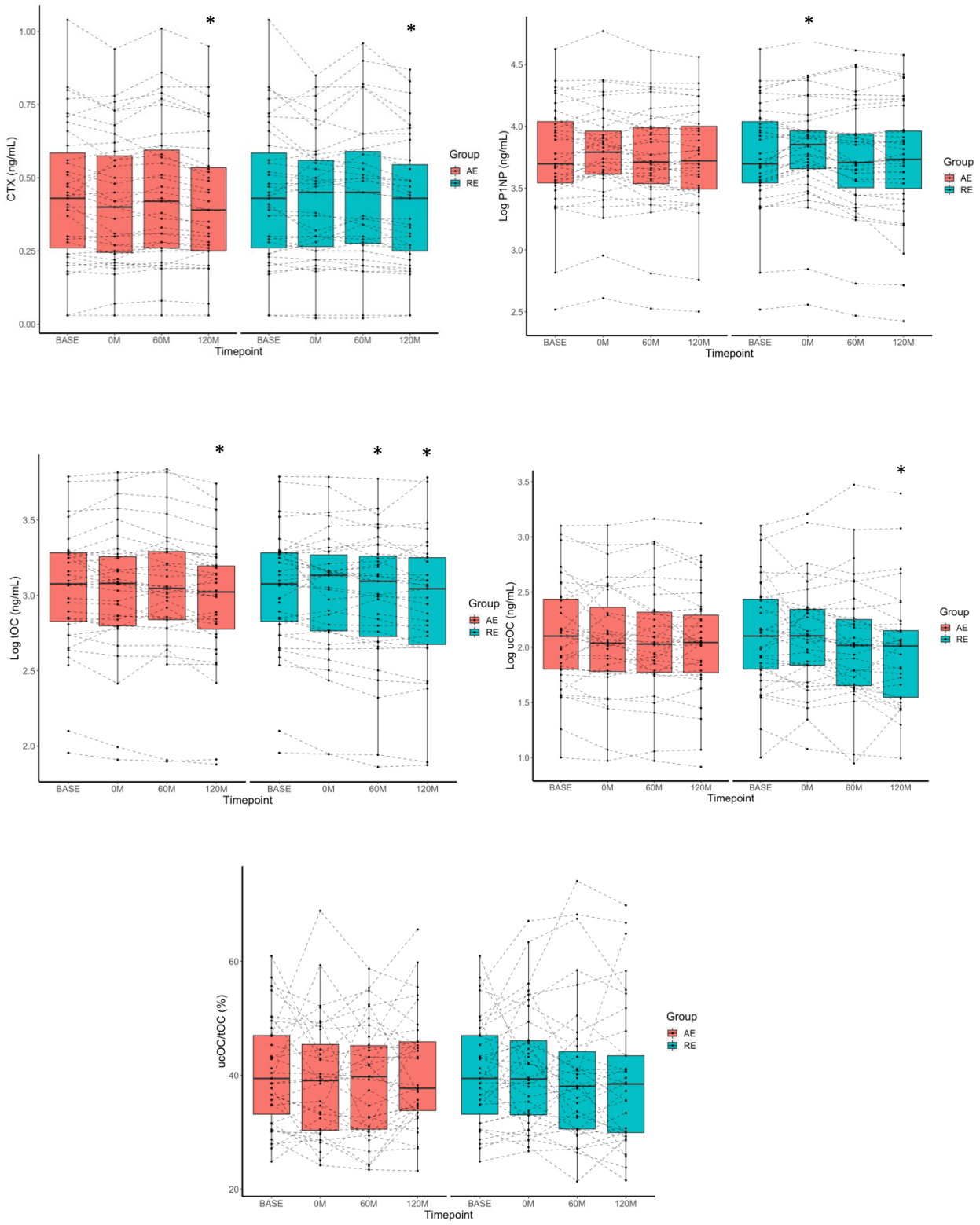

Figure 6.2 Bone remodelling markers response to acute aerobic (AE) and resistance exercise (RE) in older adults faceted by exercise mode. * Significantly different to baseline (BASE) based on the estimated marginal means of the linear mixed model, adjusted for sex.

Table 6.2 Effects of acute exercise on bone biomarkers

Independent	Predictor	Estimate	95% CI	p-value	Adj p-value
	0 min	**-0.0194**	**-0.0371, -0.0018**	**0.03***	0.09
ß-CTX	60 min	-0.0009	-0.0185, 0.0168	0.93	0.94
	120 min	**-0.0251**	**-0.0428, -0.0075**	**0.005***	**0.04***
	0 min	0.0193	-0.0163, 0.0548	0.29	0.58
Log P1NP	60 min	-0.0136	-0.0492, 0.0220	0.45	0.68
	120 min	-0.0198	-0.0554, 0.0157	0.27	0.58
	0 min	-0.0116	-0.0442, 0.0209	0.48	0.67
Log tOC	60 min	-0.0120	-0.0442, 0.0202	0.47	0.67
	120 min	**-0.0630**	**-0.0950, -0.0311**	**<0.001***	**<0.001***
	0 min	-0.0340	-0.0990, 0.0310	0.31	0.61
Log ucOC	60 min	-0.0556	-0.1195, 0.0082	0.09	0.27
	120 min	-0.0612	0.1245, 0.0021	0.06^	0.27
	0 min	-0.7879	-3.4210, 1.8451	0.56	0.61
ucOC/tOC	60 min	-1.5041	-4.0906, 1.0824	0.25	0.27
	120 min	0.1190	-2.4462, 2.6841	0.93	0.27

ß-CTX, C-terminal telopeptide of type I collagen; P1NP, procollagen of type I propeptide; tOC, total osteocalcin; ucOC, undercarboxylated osteocalcin. * significantly different to baseline p <0.05. ^ indicates nearing significantly different from baseline p <0.06. Linear mixed models were performed, and all models were adjusted for sex and adjusted for multiple comparisons.

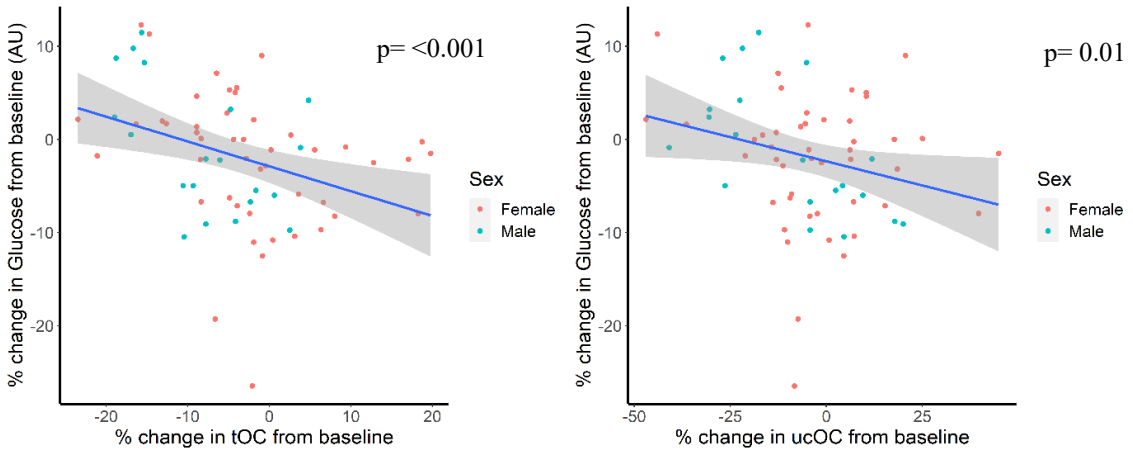

Figure 6.3 Relationship of the peak percentage change in glucose with tOC and ucOC post exercise

We then investigated the responses *within* each exercise group (**supplementary Table 2**). Following RE but not AE, P1NP increased immediately post-exercise (p= 0.02), before returning to baseline levels by 60 mins. Compared to baseline the concentration of ucOC decreased 120 mins after the RE bout (p= 0.01) but was not changed by AE .

6.4.2 Association of muscle function and bone biomarkers at baseline

Higher hand grip strength and SCP were related to higher P1NP levels at baseline (β= 0.02 ng/mL, p= 0.04, FDR= 0.05; β= 0.002 ng/mL, p= 0.005 FDR= 0.02, respectively) (see **Figure 6.4**). There was a trend towards higher LMQ also being related to higher P1NP levels (β= 0.04 ng/mL, p= 0.06, FDR= 0.07). A higher SCP was related to higher β-CTX levels (β= 0.001 ng/mL, p= 0.03, FDR= 0.04). Higher gait velocity was related to higher β-CTX levels (β= 0.46 ng/mL, p= 0.03, FDR= 0.04). Poorer physical performance (higher PPT score) was related to lower ucOC, P1NP and β-CTX levels, after adjustments for multiple comparisons the correlation with ucOC was weaker (ucOC: β= -0.04 ng/mL, p= 0.04, FDR= 0.12; P1NP: β= -0.04 ng/mL, p= 0.02, FDR= 0.04; β-CTX: β= -0.02 ng/mL, p= 0.02, FDR=0.04, respectively). TUG performance and ASM were not related to any bone biomarkers at baseline (all p>0.05).

6.4.3 Associations of baseline muscle function with bone biomarker-responses after acute exercise

Those with a higher baseline grip strength, muscle power (higher SCP) and muscle quality (higher LMQ) had a lower β-CTX exercise response (**Table 6.3)**. A higher baseline grip strength and higher muscle quality also trended towards a lower tOC exercise response (p<0.06). Poorer baseline mobility indicated by a higher time to perform the TUG was associated with a lower P1NP exercise response, but a higher β-CTX exercise response, after adjustment for multiple comparisons the correlation with P1NP was weak (P1NP: β= -0.005 ng/mL, p= 0.02, FDR= 0.10; β-CTX: β= 0.002 ng/mL, p= 0.02, FDR= 0.02). Gait velocity was not related to any of the bone biomarkers post-exercise responses.

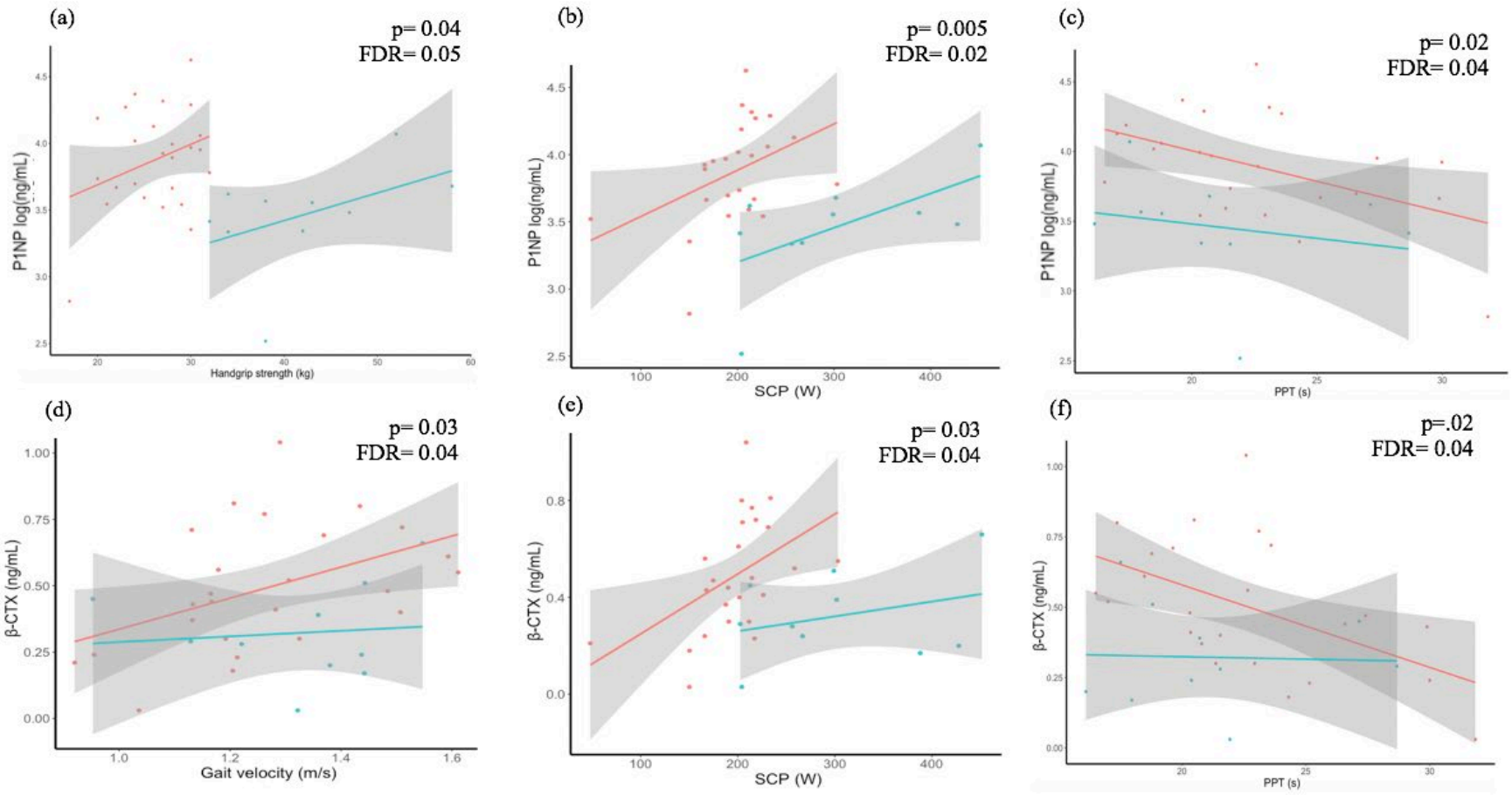

Figure 6.4 Baseline associations of muscle function with P1NP and β-CTX faceted by sex.

*p value indicates significant relationship between outcome of interest and bone turnover marker based on a beta-regression adjusted for sex. FDR is the p value after adjustment for multiple comparisons.

Table 6.3 The relationship of baseline muscle function with acute exercise responses on bone biomarkers

Independent	Muscle function * Time	Estimate	95% CI	p-value	Adj p-value
ß-CTX	velocity m/s * Time	-0.0208	-0.0434, 0.0019	0.07	0.073
	TUG* Time	**0.0024**	**0.0004, 0.0044**	**0.02***	**0.02***
	Grip strength* Time	**-0.0008**	**-0.0013, -0.0004**	**<0.001***	**0.003***
	LMQ* Time	**-0.002**	**-0.0033, -0.0006**	**0.004***	**0.007***
	SCP* Time	**-0.0001**	**-0.0001, -0.0000**	**0.001***	**0.003***
Log P1NP	velocity m/s * Time	0.0385	-0.0075, 0.0844	0.10	0.25
	TUG* Time	**-0.0048**	**-0.0088, -0.0007**	**0.02***	0.10
	Grip strength* Time	-0.0003	-0.0012, 0.0007	0.58	0.73
	LMQ* Time	0.0004	-0.0024, 0.0032	0.78	0.78
	SCP* Time	0	-0.0001, 0.0001	0.52	0.72
Log tOC	velocity m/s * Time	-0.0264	-0.0668, 0.0139	0.19	0.33
	TUG* Time	0.0009	-0.0027, 0.0045	0.63	0.63
	Grip strength* Time	-0.0008	-0.0016, 0.0000	0.05^	0.14
	LMQ* Time	-0.0023	-0.0047, 0.0001	0.06^	0.14
	SCP* Time	0	-0.0001, 0.0001	0.63	0.63
Log ucOC	velocity m/s * Time	0.0238	-0.0558, 0.1034	0.56	0.77
	TUG* Time	-0.0033	-0.0104, 0.0037	0.35	0.77
	Grip strength* Time	-0.0002	-0.0018, 0.0014	0.84	0.84
	LMQ* Time	0.0012	-0.0035, 0.0059	0.62	0.77
	SCP* Time	0.0001	-0.0001, 0.0003	0.43	0.77
ucOC/tOC	velocity m/s * Time	1.6575	-1.5700, 4.8849	0.31	0.31
	TUG* Time	-0.1567	-0.4426, 0.1293	0.28	0.31
	Grip strength* Time	0.0345	-0.0301, 0.0992	0.29	0.31
	LMQ* Time	0.1135	-0.0781, 0.3052	0.25	0.31
	SCP* Time	0.0048	-0.0027, 0.0124	0.21	0.31

ß-CTX, C-terminal telopeptide of type I collagen; P1NP, procollagen of type I propeptide; tOC, total osteocalcin; ucOC, undercarboxylated osteocalcin. Models were adjusted for group (AE and RE) and sex. * significantly different to baseline $p<0.05$. ^ indicates $p<0.06$.

6.5 Discussion

In the current study an acute bout of exercise had minimal effect or decreased bone biomarkers in older adults and this was not specific to the mode of exercise (AE or RE). Better muscle function at baseline were associated with higher bone turnover. Specifically at baseline, a higher concentration of circulating P1NP was associated with higher handgrip strength, leg power (SCP) and, better physical performance (lower PPT score). Similarly, higher ß-CTX levels was associated with higher leg power (SCP), better gait velocity and physical performance (lower PPT score). Finally, we found that the response of these BTMs to acute exercise may be influenced by underlying muscle function.

6.5.1 Muscle function and bone biomarkers at baseline

Skeletal muscle and bone play a fundamental role in enabling locomotion and movement. It is well known that alterations to mechanical loading regulate skeletal muscle and bone mass, with evidence suggesting that bone mass maintenance depends on muscle-derived mechanical loading (21, 22). It has been suggested that bone, via ucOC is involved in muscle mass maintenance and strength in mice (2-5), but this was recently challenged (6). In humans, this remains unclear (7-9), and the link between the skeleton and muscle may not be specific to ucOC, but may include other BTMs i.e CTX and P1NP (11, 12). Here we report for the first time, that CTX and P1NP, are also related to muscle function in older adults with our data suggesting that better muscle function at baseline is associated with higher BTMs. We report that better physical function is related to higher ß-CTX, P1NP and ucOC levels, although the relationship with ucOC was weak. Previously, In older females a higher ucOC/tOC was related to increased muscle strength in one study (7), and related to reduced mobility (poorer TUG performance) and increased falls risk (8). Yet, we did not observed this relationship in the current study. It is not clear why in the current study we did not observe a relationship with the ucOC/tOC ratio, but this perhaps due to the relatively small sample size compared to previous studies. While we adjusted for sex in our models, we were underpowered to examine if males and females have a different relationship between muscle function and bone biomarkers. Data in humans about the role of ucOC in muscle mass and function remains unclear due to findings being limited to association-based studies. Direct effects of ucOC on human muscle are required in future to elucidate this relationship.

6.5.2 Acute exercise effects

Irrespective of exercise mode, serum ß-CTX decreased post exercise. This is in contrast to other acute AE studies in similar cohorts that report ß-CTX and CTX either increases (32, 41) or levels were not altered by AE (42, 43). Only one other study, to our knowledge, compared AE and RE in middle-aged, postmenopausal females (44). Similarly, they report CTX decreases after RE (with impact exercise), but, CTX was not altered by moderate AE, suggesting for that study, a mode-specific response of CTX to RE. Conflicting findings to the current study may be due to study design differences, the RCT in that study was not crossover in design. The females in that study were also younger (~12 to 15 years) possibly indicating a different phase of the postmenopausal period, which is known to affect BTMs (45), and were osteoporotic/osteopenic which may alter the osteogenic effect of exercise on bone (46). Other possible explanations for conflicting findings between studies could be explained by the fragment of CTX and analysis methods used (ELISA (43), chemiluminescence (41), ECLIA (32, 42, 44)) and different post-exercise blood sampling protocols may detect different time-course responses. Another factor that may create contracting results is whether the participants fasted or not prior to the trial as it is known that a meal can decrease BTMs (42). Given that ß-CTX is suggested to reflect bone resorption, and we report it decreases with acute exercise, it may indicate a tip in favor of the balance of (reduction in bone remodeling), and resultant over time, reduction in bone loss, however, exact mechanisms of this is poorly understood and warrant further investigation.

Regardless of exercise mode, we show acute exercise decreased tOC (by 6.5%), with no change in ucOC. When we investigated each exercise mode individually, RE decreased levels of ucOC at 120 mins compared to baseline, with no change observed after AE. This is in contrast to other studies in older adults, some reported tOC increases following AE (47-49) and impact only (jumping) exercise (50), others that tOC is not altered after AE or RE (32, 42, 43, 51, 52). Measurement of tOC only however, limits our understanding of this hormone in general(53). OC exists in the circulation in two forms, which are suggested to reflect different underlying biological processes with ucOC considered bioactive with endocrine-like effects (54-57).

Indeed, we previously showed in two separate studies that while AE did not alter tOC levels in middle-aged males and females, ucOC was increased (32, 42). Only few

studies to our knowledge, have measured ucOC following acute exercise in similar cohorts. We report previously that ucOC increases with moderate and high intensity AE (32, 42, 47) but, was not altered by RE (using the same protocol as the current study) (47). Conflicting data between that study (47), and the current could be related to different ucOC assay methods (ECLIA vs automated immunoassay following hydroxyl-apatite slurry), the males were also younger (~22 years) and obese. It is not clear why a similar increase in ucOC was not observed in the current study. Increasing age is related to an altered hormonal status, particularly in postmenopausal females (45) which may influence BTM responses. Indeed, we previously showed that the increase in ucOC following acute exercise was also related to the insulin sensitizing effects of exercise (32). In the current study, acute exercise did not alter ucOC or glucose levels, however the change in tOC and ucOC following acute exercise was related to the change in glucose, suggesting some evidence for a link between OC and glucose metabolism. Older adults are also known to be characterized by altered muscle quality and metabolism and increased risk for insulin resistance (58, 59). Whether this may be one explanation for the conflicting findings warrants further exploration.

6.5.3 Muscle function and bone biomarkers: acute exercise responses

Given the close link between bone and muscle, we also investigated whether baseline muscle strength or function is related to the acute exercise response of bone biomarkers. Our data show that those with higher muscle strength (grip strength) had lower post-exercise ß-CTX levels, tOC also trended towards a lower post-exercise response. Similarly, higher leg power (SCP) and muscle quality (LMQ) was related to lower ß-CTX levels, whereas poorer mobility (slower time to perform the TUG) was related to an increase in ß-CTX. Taken together, this suggests that underlying muscle health status may be related to circulating levels of BTMs post exercise or in other words, those with lower muscle strength at baseline had a greater change in BTMs post-exercise responses. This observation is similar to previous reports in other diseases, such as type 2 diabetes where it was reported that those with higher glucose and HbA1c (poorer glucose metabolism) at baseline exhibit a greater reduction in glucose and HbA1c after exercise (60). In context to the current study, particularly ß-CTX, whereby greater baseline muscle strength, leg power and mobility were related to lower post-exercise levels as discussed earlier this may indicate a reduction in bone remodeling, but, this

warrants further research. Altogether our data suggest that the link between muscle function and BTMs includes commonly used bone biomarkers, and these could represent a biomarker for detecting a change in muscle function in older adults. These findings need to be confirmed in larger, prospective cohort studies.

6.5.4 Future directions

Comparing data across acute exercise studies is challenging due to factors influencing responses. Based on our understanding of bone loading, bone responds to loads of novel and dynamic distributions, suggesting bone should optimally load to RE, not AE particularly in a low gravitational environment i.e. cycling. However, our results suggest that load and gravity may not be the only factors affecting bone biomarker response, at least to acute exercise. Sex was significant in the analysis models, and explained some of the variability in ß-CTX, tOC and P1NP, indicating sex differences in the levels of these biomarkers. However, due small sample of males we were unable to examine whether responses were sex-specific. We previously demonstrated, albeit, in young adults that tOC and ucOC responses to high intensity AE were not sex-specific (61). However, recent review articles discuss evidence, potential mechanisms and physiological reasons for differences in sex-specific adaptations to exercise (62, 63). Whether there are sex-specific responses of bone biomarkers to acute exercise in older adults remains unclear, and should be explored. It is possible that the rapid change in bone biomarkers observed is not a reflection of altered bone turnover *per se*. The fluid shifts occurring within bone in response to exercise may alter the rate these proteins are released into the circulation, or that bone biomarkers are released during exercise from other organs, i.e. liver (64, 65). However this is unlikely for those used in the current study. Other potential metabolic factors, i.e. reactive oxygen or nitrogen species, acidosis or serum calcium availability may also be involved (66-68). Precise mechanisms should be explored in future to elucidate the acute exercise effects on bone biomarkers in general. Lastly, while our data aligns with some but not all findings, further studies are required to understand the complex relationship of bone biomarkers with different exercise intensities or modes and adults in different stages of life (i.e in females pre- peri- or post-menopause). Future studies should also consider multiple sampling timepoints over an extended period of time, and larger, randomized controlled studies are required.

6.5.5 Limitations

This study includes community-dwelling older adults who did not have diabetes. Although the sample size was small, the results of this study should be considered hypotheses generating. The participants in this study were taking anti-cholesterol, Vitamin D and antidepressants, which may have affected the relationships (69, 70), however, this study was cross-over in nature in an attempt to control for such confounders. The strengths of this study were its crossover design, and control procedures including performing testing visits in a fasted state, performed in the morning and at the same time in an attempt to control for diurnal variation. The males and females were combined in all analyses and sex was adjusted for in all statistical models, but this may limit the correlations. However, due to the low sample size, we were underpowered to perform additional analyses stratified by sex. The study did not include control data; therefore, the acute exercise effects on these BTMs may also be influenced by diurnal variation although we tested all participants in the morning and following an overnight fast to minimize these effects.

6.5.6 Conclusion

We demonstrate in older adults that the relationship between muscle quality/function and BTMs is not specific to ucOC, but to BTMs in general. Furthermore, a higher circulating levels of BTMs are linked to better muscle function. Exercise (aerobic or resistance exercise) had minimal effects on BTMs and perhaps even reduced the levels of β-CTX and total OC. The results of this study may potentially strengthen the evidence for a bone-muscle interaction axis, however this needs to be explored and confirmed in future mechanistic studies.

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6.7 Supplementary materials

Supplementary Table 1. Effects of acute exercise on BRMs: Results of the linear mixed model adjusted for sex

	Predictor	Estimate	CI	P-val
ß-CTX	**Sex**	**-0.1759**	**-0.3324 – -0.019**	**0.028***
	Group * 0 mins	0.0029	-0.0221 – 0.0278	0.823
	Group * 60 mins	0.0026	-0.0224 – 0.0275	0.840
	Group * 120 mins	-.0031	-0.0281 – 0.0218	0.805
P1NP	**Sex**	**-0.4205**	**(-0.7030 – -0.1381)**	**0.004****
	Group * 0 mins	0.0355	(-0.0148 – 0.0858)	0.167
	Group * 60 mins	0.0017	(-0.0486 – 0.0520)	0.947
	Group * 120 mins	-0.0118	(-0.0621 – 0.0385)	0.646
tOC	**Sex**	**-0.3115**	**(-0.6012 – -0.0219)**	**0.035***
	Group * 0 mins	-0.0137	(-0.0595 – 0.0321)	0.557
	Group * 60 mins	-0.0324	(-0.0778 – 0.0130)	0.162
	Group * 120 mins	0.0004	(-0.0452 – 0.0460)	0.987
ucOC	Sex	-0.3125	-0.6493 – 0.0243	0.069
	Group * 0 mins	0.0155	-0.0756 – 0.1066	0.738
	Group * 60 mins	-0.0156	-0.1055 – 0.0743	0.733
	Group * 120 mins	-0.0384	-0.1287 – 0.0519	0.404
ucOC/tOC	Sex	0.0568	-6.8135 – 6.9270	0.987
	Group * 0 mins	1.2949	-2.3964 – 4.9862	0.492
	Group * 60 mins	1.0875	-2.5553 – 4.7304	0.559
	Group * 120 mins	-0.8089	-4.4680 – 2.8502	0.665

Linear mixed models were performed and all models were adjusted for sex. ß-CTX, C-terminal telopeptide of type I collagen; P1NP, procollagen of type I propeptide; tOC, total osteocalcin; ucOC, undercarboxylated osteocalcin.

Supplementary Table 2. Within group time point responses- Estimated marginal means from the linear mixed model adjusted for sex.

		Aerobic		Resistance	
Independent	**Time point**	**EMM**	**CI**	**EMM**	**CI**
CTX	BASE	0.405	0.323 - 0.487	0.405	0.323 - 0.487
	0m	0.385	0.303 - 0.467	0.388	0.306 - 0.470
	60m	0.404	0.322 - 0.486	0.407	0.325 - 0.489
	120m	**0.380**	**0.298 - 0.462*^**	**0.377**	**0.295 - 0.459*^**
Log P1NP	BASE	3.67	3.52 - 3.81	3.67	3.52 - 3.81
	0m	3.69	3.54 - 3.83	**3.72**	**3.57 - 3.87***
	60m	3.65	3.50 - 3.80	3.65	3.51 - 3.80#
	120m	3.65	3.50 - 3.79	3.63	3.49 - 3.78#
Log tOC	BASE	2.97	2.81 - 3.12	2.97	2.81 - 3.12
	0m	2.95	2.80 - 3.11	2.94	2.79 - 3.09
	60m	2.95	2.80 - 3.11	**2.92**	**2.77 - 3.07***
	120m	**2.90**	**2.75 - 3.05*#^**	**2.90**	**2.75 - 3.06***
Log ucOC	BASE	2.03	1.86 - 2.21	2.03	1.86 - 2.21
	0m	2.00	1.82 - 2.18	2.02	1.84 - 2.20
	60m	1.98	1.80 - 2.16	1.96	1.78 - 2.14
	120m	1.97	1.79 - 2.15	**1.94**	**1.76 - 2.11***
ucOC/tOC	BASE	40.5	36.5 - 44.4	40.5	36.5 - 44.4
	0m	39.7	35.7 - 43.6	41.0	37.0 - 44.9
	60m	39.0	35.0 - 42.9	40.0	36.1 - 44.0
	120m	40.6	36.7 - 44.5	39.8	35.8 - 43.7

EMM= estimated marginal mean based on the linear mixed-effects model adjusted for sex. Results are averaged over the levels of sex. Confidence interval used: 0.95. P-value adjustment: Tukey method

*indicates significantly different from baseline p=<.05

# indicates significantly different from time point 0m p=<.05
^indicates significantly different from time point 60m p = <.05

Chapter 7: General discussion and conclusions

7.1 Major findings

Older adults are at high risk for sarcopenia and osteoporosis, which in turn can lead to falls, fractures and early mortality (78, 88, 94). Given the ageing population and increases in sedentary behaviours, sarcopenia prevalence and burdens are predicted to rise. Bone and muscle are closely linked and tightly regulated by mechanical load such as exercise. Yet, it is not clear which bone-derived hormones are involved in this crosstalk. The ucOC hormone is involved in glucose regulation and possibly muscle mass maintenance and strength, at least in rodents. Consequently, it was suggested that ucOC may have potential as a therapeutic target to treat metabolic disorders including insulin resistance and T2D, as well as muscle wasting. However, the data linking ucOC with glucose regulation, and in particular with muscle mass and strength in humans, are contradictory. Furthermore, given that BTMs are released by bone during remodelling, and BTM levels are altered by exercise, it can be hypothesised that BTMs might be involved in bone-muscle interaction. As such, the major aim of this thesis was to explore bone-muscle interaction by examining BTMs, with a focus on ucOC, and their relationship with muscle function in older adults.

To address these aims four studies were conducted**. Study 1** characterised for the first time the effect of ageing on OC forms and ratios in humans. This was a crucial step to understand OC changes across the lifespan in general. In **Study 2**, I performed a longitudinal analysis of the relationship between ucOC with physical function, including long term injurious falls risk over 15 years. This was an important next step to strengthen previous cross sectional based evidence linking ucOC to muscle function in humans. In **Study 3**, a systematic review was performed to determine how BTMs respond to acute exercise, given that exercise is the cornerstone approach to improve musculoskeletal health in all ages including older adults. It was important to investigate whether responses are specific to exercise mode, intensity, age and sex. Lastly, this thesis included a randomised crossover clinical exercise trial (**Study 4**) in an attempt to identify whether ucOC and BTMs are related to muscle function in older adults. The major novel findings of this thesis are listed below (and illustrated in **Figure 7.1**):

i. Reference ranges (95% reference limits) for all OC forms and ratios across the adult male lifespan were defined **(study 1).** Circulating tOC, ucOC and cOC levels

follow a U-shape pattern with increasing age, whereas the ucOC/tOC ratio increases, and cOC/tOC decreases, indicating that the ucOC/tOC ratio is a good measure of the ageing effect on OC, and therefore perhaps has greater clinical utility.

ii. In older women, a higher ucOC/tOC ratio was related to poorer physical function, including its long term decline, and injurious falls risk over 15 years (**Study 2**). The ratio was also sensitive to detect those older women that had a fear of falling. This study, in combination with **Study 1,** provided evidence of the potential clinical utility of the ucOC/tOC ratio as a biomarker to identify individuals at risk for a loss of physical function and increased falls risk.

iii. **Study 3** revealed the limited number of studies that have explored the effects of acute exercise on BTMs in middle-aged and older adults, highlighting a major gap in our understanding of acute exercise responses on bone biomarkers in older adults.

iv. In **study 4**, I showed that exercise has minimal effect, and may even decrease ß-CTX and tOC. I hypothesise that alteration to bone biomarkers following acute exercise may not reflect bone turnover *per se*, but may be a result of other metabolic processes.

v. In the cross-sectional analysis of older adults (**study 4**), I identified that higher baseline BTMs was correlated with better baseline muscle function, suggesting that the link between muscle function and bone may not be specific to ucOC but to BTMs in general.

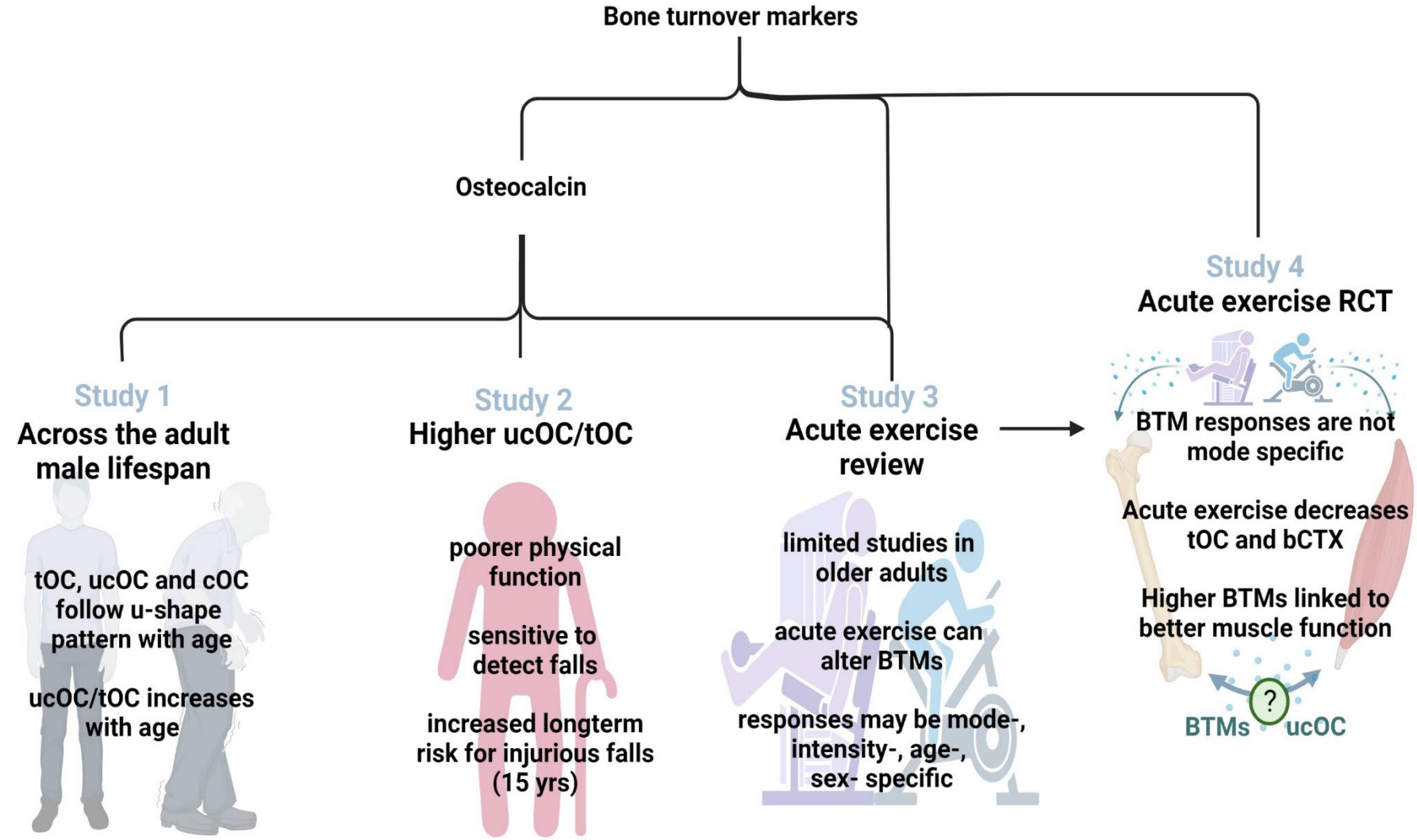

Figure 7.1 Summary of the major findings of this thesis. Created with BioRender.com

7.1.1 Osteocalcin in ageing

7.1.1.1 *The ucOC/tOC ratio increases with age and is related to poorer physical function and falls*

Results from this thesis provide evidence that measurement of tOC alone, as performed in many studies and clinically, limits our understanding of OC in general. The measurement of ucOC should also be considered and expressed as ucOC/tOC, which may have better clinical utility. Currently, the wide use and measurement of ucOC in a research setting is limited by the assay technique, no gold-standard or optimal method to measure ucOC exists, with the current methods all having limitations. Only the HAP method allows the expression of ucOC/tOC ratio, yet this method is highly dependent on technical details. Future steps for the potential usefulness of ucOC/tOC as a clinical biomarker would require the development of an automatic and reliable assay to detect ucOC, with the capability to express the ucOC/tOC ratio. Many available assays require running two independent assays on tOC (GLA-OC) and ucOC (GLU-OC) components, therefore the expression of ucOC as a ratio of tOC is not possible. Before being incorporated into standard clinical practice, future studies should determine age-based reference ranges in larger more heterogeneous cohorts, performed for example in both sexes and clinical populations.

I demonstrated that the ucOC/tOC ratio, but not ucOC alone, is related to falls and falls-related hospitalisation. I also reported that the ucOC/tOC ratio is higher in women who had a fear of falling, this suggests that a fall had probably already occurred. My findings also suggest that ucOC/tOC may be a representative clinical biomarker, where no such biomarker currently exists, to detect individuals at high risk of a decline in physical function. This may enable interventions to begin early and possibly prevent hazard outcomes such as falls, at least in older females. These findings should be confirmed in males. Future research such as interventional studies that can demonstrate adaptation in muscle function with changes in the ucOC/tOC ratio and a reduction in falls are required.

7.1.2 Acute exercise and muscle function

7.1.2.1 *The acute responses of BTMs following exercise are not mode specific in older adults*

Despite the known beneficial effects and role of exercise in maintaining musculoskeletal health, particularly in older adults who are at high risk for sarcopenia and osteoporosis, data from this thesis demonstrate that there is large under-representation of older adults in acute exercise studies that investigate BTMs. The evidence suggest that BTM responses to acute exercise may be specific to exercise mode, intensity, age and sex (**study 3**) (Figure 7.2).

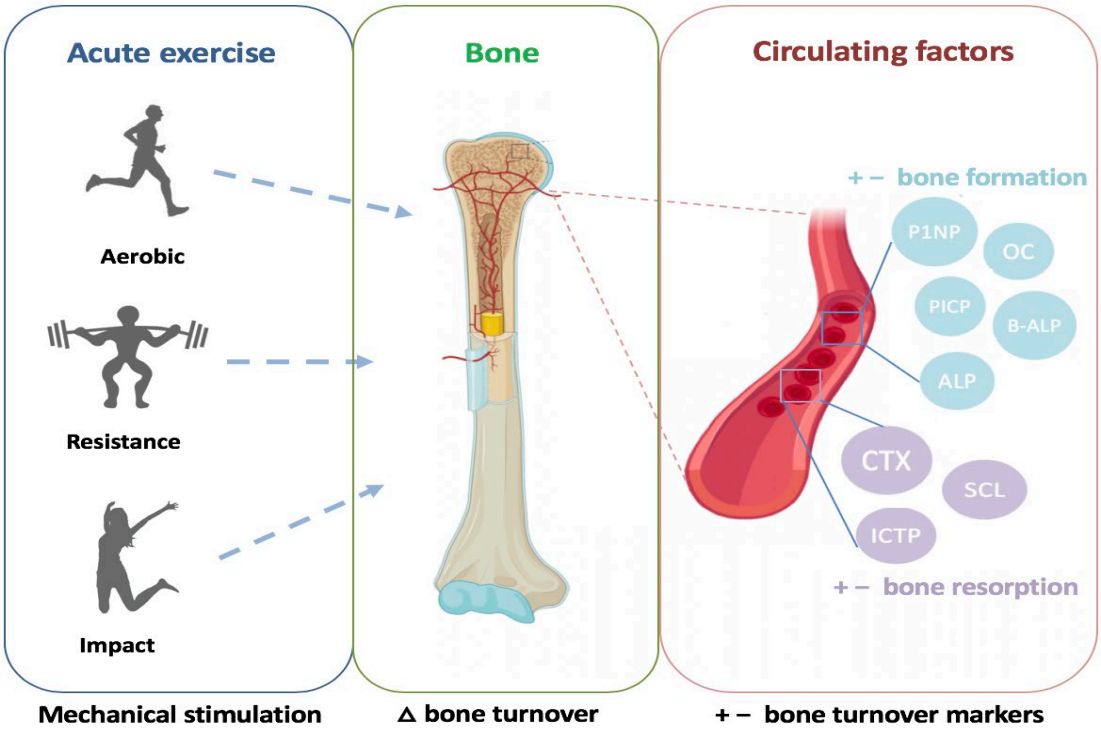

Figure 7.2 A schematic representation of the effects of acute exercise on BTMS reported in the exercise literature.

In **study 4**, the data demonstrate that there was no difference in BTM responses based on the mode of exercise. Based on the founding principles to optimise bone loading (258, 524-526) and given that AE was performed in a low-gravitational environment (cycling) versus the power RE and jumping regimen, this was a surprising finding. My data suggest that load and gravity may not be the only determining factors of these particular bone biomarker responses to acute exercise. It is possible that exercise at different intensities and duration may elicit different BTM responses, possibly related to different recruitment of muscle fibres or substrate metabolism (such as aerobic or anaerobic energy metabolism) (555). I used moderate intensity AE and RE (**study 4**) and as such studies using a higher intensity are needed (30, 427). It is also possible that the rapid change that we and others observe following acute exercise may not reflect

underlying bone turnover *per se*. It could be related to the fluid shifts within bone that alter the rate at which proteins are released (556). Some BTMs are also produced by other organs (557), but this is unlikely for the markers we studied in this thesis. Other metabolic factors could also be involved i.e. reactive oxygen or nitrogen species, acidosis or serum calcium availability (558-560). Finally, it is known that exercise shifts plasma volume, although whether this is also a confounding factor on circulating BTMs is unclear. Further larger RCTs are required that explore whether BTM responses are different based on different exercise intensities and duration in this population. Future studies should also measure plasma volume at baseline and during post-exercise blood sampling time points to account for exercise related fluid shifts on BTM levels. In addition, metabolic factors should be explored as a potential confounder on the BTM responses.

7.1.2.2 *Muscle function is linked to BTMs*

Although this thesis performed a longitudinal study (**study 2**) strengthening the evidence of an association between ucOC/tOC with physical performance and falls risk, we did not assess the relationship with other BTMs. **Study 4** reported that BTMs (P1NP and β-CTX), not ucOC, were linked to better muscle function. One explanation for the conflicting data between **study 2**, which supports the link between ucOC and muscle function, and **study 4**, which demonstrated no link, is the limited sample size in **study 4**. Despite this, data from this thesis strengthens the link between BTMs and muscle function. However, larger RCTs are required to confirm our findings, which should be considered as hypothesis-generating.

Based on the findings of this thesis and on the understanding of bone metabolism, it is clear that bone remodelling and bone turnover in ageing is a complex process that is probably dependent on many factors i.e. population, sex and age, which could explain some of the variability of results in the literature. In addition, the link between BTMs and muscle function may also involve other factors such as skeletal mechanoreceptors, the effects of comorbidities, medications, poor nutrition, vitamin D deficiency, secondary hyperparathyroidism, and impaired renal function (561-563). Assessment of these was beyond the scope of this thesis. Whether BTMs could be used to identify those at risk of low muscle function and falls is yet to be studied and will require much larger longitudinal cohort studies.

7.2 Limitations and suggestions for future research

Specific limitations for each study are described in the relevant sections (**chapter 3 to 6**). General limitations of this thesis are described below.

1. **Study 1** reported age-reference ranges of OC forms and ratios for adult males. As such, the findings cannot be generalised to females. Similarly, **Study 2** included only older females. Therefore, the relationship of ucOC with physical function and falls may be different in males. Future research should replicate these studies in both sexes
2. In **Study 3** I performed a systematic review rather than a meta-analysis as the number of studies identified was low and the studies used different outcomes, blood sampling methods and exercise protocols. In future, when more studies become available in this population, this data should be meta-analysed to confirm magnitude and direction of BTM responses as well as delineate factors contributing to this relationship i.e. sex, age, body mass index, exercise protocol characteristics.
3. In **Study 4** I only used one baseline blood sample for both study visits in order to reduce the number of blood samples taken during the trial (n=9 blood samples, which was approximately 150mL per visit). It is possible that baseline levels of BTMs may be different on different days. I have tried to reduce this risk by having all trials performed in the morning in a fasting state. In addition, there are many metabolic factors that may be involved in the alteration of circulating BTM levels in response to acute exercise, these should be investigated.
4. Moderate intensity AE and RE was used in **study 4,** this may be too low to examine responses of bone and muscle which may in part explain a lack of change in BTMs. Future studies using high intensity AE and RE are needed to clarify these findings.
5. OC is vitamin K dependent, yet vitamin K was not assessed in the current thesis. Thorough dietary and nutrient analysis as a potential confounder of OC and potentially other BTMs should be performed in future studies.
6. The data supporting a bone-muscle interaction in **Study 2** and **study 4** are observational in nature, and are not evidence of a direct cause-effect of this relationship. It will be important to investigate directly the cause-effect

relationship between ucOC and muscle mass regulation or muscle function, and the mechanisms of action and signalling pathways involved.

7. Lastly, the relationship suggested between increased BTMs and higher muscle function (**study 4**) is correlative in nature and needs to be confirmed in a much larger cohort, across different populations (age groups, clinical populations) and the relationship explored in a longitudinal design.

7.3 General conclusions

In conclusion, this thesis adds significant new knowledge supporting the growing body of evidence for bone-muscle interaction via ucOC and potentially BTMs.

- The ucOC/tOC ratio increases with increasing age, and a higher ucOC/tOC is related to poorer physical function and long term injurious falls risk. Suggesting that ucOC/tOC ratio could be used to identify those at high risk for a decline in physical function and falls. Future research should measure ucOC to build our understanding of this hormone in human health, and across clinical populations i.e. T2D and sarcopenia.
- Besides ucOC, this thesis also uncovered a potential link between BTMs in bone-muscle crosstalk. While this data need to be confirmed in larger studies, for translation of this thesis findings, this may have important clinical utility. Given BTMs are already used clinically, it would be an easy to implement strategy into current clinical practice.
- Acute exercise can alter BTM levels. The variability in responses may be explained partly by sex. The acute exercise effects observed on BTMs may not reflect underlying bone turnover *per se*, but possibly a consequence of metabolic and other processes which are poorly understood and currently under explored.

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Appendices

Appendix 1 Published version of manuscript: Sarcopenia definition: Does it really matter? Implications for resistance training.

Ageing Research Reviews 78 (2022) 101617

Contents lists available at ScienceDirect

Ageing Research Reviews

journal homepage: www.elsevier.com/locate/arr

Sarcopenia definition: Does it really matter? Implications for resistance training

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ARTICLE INFO

Keywords:
Sarcopenia
Exercise
Ageing
Older adults
Clinical populations

ABSTRACT

The loss of muscle mass, strength and function, known as sarcopenia, is common in older adults, and is associated with falls, fractures, cardiometabolic diseases, and lower quality of life. Sarcopenia can also occur secondarily to chronic diseases. Recently, sarcopenia was recognized as a disease with an International Classification of Disease (ICD) code, yet, at least five definitions for its clinical identification exist. Most definitions include three themes: low muscle mass, strength and physical performance. However, the definitions vary by the number of themes needed to diagnose sarcopenia and, within each theme various parameters and cut-off levels exist. The lack of consensus on what constitutes a diagnosis can create confusion and hesitation in sarcopenia diagnosis. Currently, no pharmacological treatment exists for sarcopenia. Resistance training (RT) is safe and effective to improve muscle mass, strength and physical performance in older adults and clinical populations. Based on current guidelines, whether an individual is defined as "sarcopenic", or not, does not change the way RT is prescribed. Here, we present evidence and the inconsistencies in sarcopenia definitions and recommend that focus should be on optimizing ways to prescribe RT and increase long-term adherence, rather than on slight modifications to sarcopenia definitions.

1. Sarcopenia and its definitions: scope of the problem

Older adults can now expect to live to over 80 years of age (Woessner et al., 2021). However, increases in life years does not always translate to healthy life years. Rather, it is commonly accompanied by disability, increased risk for chronic disease and a poor quality of life (Kennedy et al., 2014). The loss of muscle mass and strength and/or physical function is known as sarcopenia, depending on the clinical definition used to identify it (Bhasin et al., 2020; Chen et al., 2020; Cruz-Jentoft et al., 2019; Fielding et al., 2011; Studenski et al., 2014). Sarcopenia is common in older adults (> 65 years) with estimated prevalence varying between 10% and 50%, large variability in prevalence is contingent on the definition used (Cruz-Jentoft et al., 2014; Shafiee et al., 2017). Sarcopenia is commonly associated with a higher risk of falls and fractures, reduced capacity to perform activities of daily living (ADLs) and a loss of independence (Beaudart et al., 2017; Zhang et al., 2018). It is a multifactorial disease, and some of its risk factors include age, sex, low level of physical activity, poor diet and, chronic inflammation. As such, sarcopenia often develops in conjunction with presence of cardiometabolic disease (Collamati et al., 2016; Mesinovic et al., 2019). Sarcopenia was recognized as a disease with its own International Classification of Disease, ICD-10 code (M62.84)(Anker et al., 2016). Although one can hypothesize that this new ICD code will promote screening for sarcopenia and therefore treatment and management, there is no consensus on its diagnostic criteria. This lack of agreement in the cut-off criteria to diagnose sarcopenia between organizations, clinicians and researchers limits the use of an ICD code, potentially complicating the effective management of the disease.

Currently, there is no universally accepted definition for sarcopenia. The validity and predictive values for adverse outcomes based on the available definitions is varied (Levinger and Duque, 2021; Sim et al., 2019a, b). There are at least five definitions used to diagnose sarcopenia including the European Working Group on Sarcopenia in Older People (EWGSOP2) (Cruz-Jentoft et al., 2019); the Foundation for the National

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https://doi.org/10.1016/j.arr.2022.101617
Received 5 November 2021; Received in revised form 21 January 2022; Accepted 30 March 2022
Available online 1 April 2022
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Institutes of Health (FNIH) (Studenski et al., 2014); Asian Working Group for Sarcopenia (AWGS) (Chen et al., 2020); Sarcopenia Definitions and Outcomes Consortium (SDOC) (Bhasin et al., 2020); and the International Working Group on Sarcopenia (IWGS) (Fielding et al., 2011). Of these, three represent updates to original definitions (Chen et al., 2014; Cruz-Jentoft et al., 2010; Studenski et al., 2014).

The criteria used to identify sarcopenia according to the five most commonly used definitions can be generally categorized into three main themes: a) muscle strength, usually hand grip strength, an assessment of upper limb strength (four of the five definitions) which has shown to have the capacity to identify older adults at risk for falls and fractures (Cöster et al., 2020; Karlsson et al., 2012; Rosengren et al., 2012); b) muscle mass, usually appendicular skeletal muscle mass adjusted to height or body mass index (BMI) (four of the five definitions) and c) physical performance i.e. gait speed, an assessment of mobility that has been associated with survival and predicts incident disability in older adults (four of the five definitions) (Perera et al., 2016; Studenski et al., 2011). As seen in Fig. 1, even within these three themes, there are a diverse range of acceptable parameters included in each definition which may measure a different muscle characteristic. For example, muscle strength defined by EWGSOP2 includes hand grip strength, or the 5-time chair stand, an easy, portable assessment of lower limb muscle power, which has been shown to be associated with falls, frailty, slowness and functional limitation in activities of daily living in older adults (Alcazar et al., 2018; Baltasar-Fernandez et al., 2021; Shea et al., 2018; Ward et al., 2015). Of note, even among those definitions that include the same muscle parameter, different cut-off values are used (Fig. 1). To our knowledge, there are also working groups currently formulating new definitions for sarcopenia, some of which suggest adding additional diagnostic measures including calf circumference (Mo et al., 2020), muscle density assessed by computed tomography (CT) (Wang et al., 2020b), hand grip strength asymmetry (Parker et al., 2020) and perhaps even lip, tongue and suprahyoid muscle strength (Abe et al., 2020; Yamaguchi et al., 2020). The reality of numerous definitions for sarcopenia existing (including possibly more to come) together with a lack of agreeance on cut-off levels for individual muscle parameters,

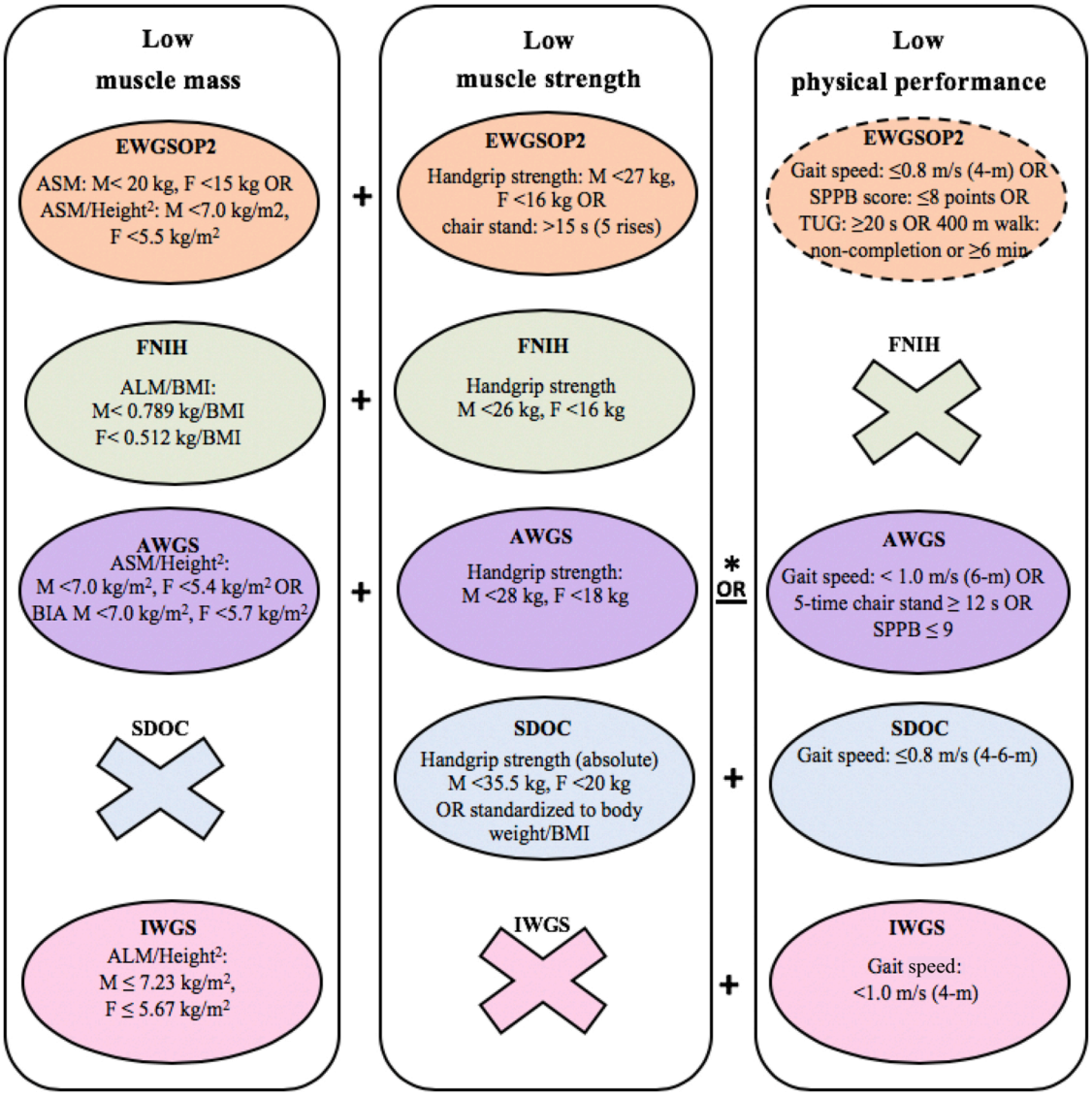

Fig. 1. Most commonly used definitions for sarcopenia: EWGSOP2 (European Working Group on Sarcopenia in Older People); FNIH (Foundation for the National Institutes of Health); AWGS (Asian Working Group for Sarcopenia); SDOC (Sarcopenia Definitions and Outcomes Consortium), IWGS (International Working Group on Sarcopenia). Abbreviations: M = male; F = female; ASM = appendicular skeletal muscle mass; SPPB = short physical performance battery; TUG = timed up-and-go; DXA = dual-energy X-ray absorptiometry Dashed line in EWGSOP2: compromised physical performance is used only to determine severity of sarcopenia once sarcopenia has already been diagnosed. *If all three themes are identified as compromised then this would identify severe sarcopenia based on the revised AWGS [13].

leads to confusion in its diagnosis in both clinical and research settings. Inconsistent reports in the literature related to prevalence of sarcopenia are shown largely to be explained by the definitions chosen. In other words, when different definitions and cut off points are used, different results are obtained (Grosicki et al., 2020; Petermann-Rocha et al., 2019; Sim et al., 2019a, b). Not only does this lead to variable and inconsistent reports, it may also contribute to hesitation in a clinical setting to diagnose a patient as "sarcopenic". The complexity in reaching an agreeable definition maybe, at least in part, be due to the fact that older adults with sarcopenia, similar to frailty, are characterized as a very heterogeneous group, which may have some practical challenges clinically (Cesari and Kuchel, 2020). However, it is not just the definition that is important to identify sarcopenia, but also which health professional/s is/are responsible for its diagnosis (Yeung et al., 2020). For example, dieticians, exercise physiologists and physiotherapists should all play a role. An important question that remains is whether the use of a different clinical definition for sarcopenia changes the negative outcomes associated with its progression. This should be explored in future studies.

Discrepancies between agreeable cut off levels and parameters used to define sarcopenia raise an important clinical question: *is having a precise cut-off level for the diagnosis of a patient as "sarcopenic" critical for disease management?* A specific diagnostic cut-off level is undeniably pivotal for the accurate prescription of pharmacological treatment and is also valuable from a patient perspective as increased knowledge can increase self-empowerment (Wurcel et al., 2019). As stated by Cesari and Kuchel (2020) "we must not let the perfect become the enemy of the good." In other words, additional parameters and definitions or criteria for sarcopenia may complicate and possibly hinder the prescription of the only known effective treatment for sarcopenia, specifically, a lifestyle approach incorporating progressive resistance training (RT) in conjunction with a healthy diet comprising adequate protein and energy intake (Dickinson et al., 2013; Fiatarone et al., 1994; Tieland et al., 2012).

2. Sarcopenia: what is our best defense?

One of the most consistent changes with advanced age is the decline in skeletal muscle mass and strength. Skeletal muscle comprises ~40% of the human body weight, and its functions are widespread, including postural, mobility, energy storage and metabolism. Longitudinal data clearly demonstrates a decline in muscle mass, muscle strength and power beginning ~35 years of age (Frontera et al., 2000). Notably, muscle strength and power decline to a greater extent than muscle mass, accounting for much of the disability and functional limitations associated with these age-related changes and not muscle mass, *per se* (Frontera et al., 2000; Goodpaster et al., 2006).

Sarcopenia often presents as a comorbidity of other cardiometabolic diseases and has common risk factors (increasing age, physical inactivity, chronic inflammation and malnutrition) (Booth et al., 2012; Collamati et al., 2016; Mesinovic et al., 2019; Pacifico et al., 2020). Many of these chronic cardiometabolic diseases (such as cardiovascular disease, type 2 diabetes, and others) also share the skeletal muscle biological characteristics of sarcopenia with alterations to muscle size (fiber number and atrophy of type II fibers, motor units), increased fat infiltration, decreased capillarization, chronic inflammation, increased oxidative stress and insulin resistance, of which, exercise has been shown to effectively mitigate (Furman et al., 2019; Kalyani et al., 2014; Moylan and Reid, 2007; Suzuki et al., 2018). Notably, some of the loss of muscular power and function experienced by older adults and clinical populations can be attributed to age-related neuromuscular loss (Hunter et al., 2016).

There are currently no approved pharmacotherapies for the treatment of sarcopenia. Phase 2 clinical trials testing the effect of an anti-myostatin antibody showed minimal effect on muscle function (Becker et al., 2015). The only intervention that consistently shows to improve muscle mass, strength, and physical function in older adults is exercise training and particularly progressive resistance training (RT) (Bårdstu et al., 2020; Henwood et al., 2008; Kalapotharakos et al., 2007, 2005; Kirk et al., 2019a; Mertz et al., 2021). Progressive RT is a safe and effective approach to attenuate, and in some cases reverse the age-related loss of muscle mass and strength (Dent et al., 2018; Fragala et al., 2019; Landi et al., 2014).

3. Resistance exercise: the front line defense

Progressive RT is considered a first-line strategy to prevent and manage sarcopenia (Dent et al., 2018; Fragala et al., 2019; Izquierdo et al., 2021). It provides numerous benefits including increasing muscle mass, strength, endurance, power and physical function, and lowering the risk of falls and associated injury such as fracture (Bårdstu et al., 2020; Fiatarone et al., 1990; Henwood et al., 2008; Kalapotharakos et al., 2007, 2005; Liu-Ambrose et al., 2004; Skelton et al., 1995). These are essential muscle characteristics which are required to perform ADLs in older adults and clinical populations (Kraemer et al., 2001; Pollock et al., 2000; Wang et al., 2020a). RT is also consistently demonstrated to be safe, effective and recommended for almost all populations including healthy older adults and those with chronic diseases such as cardiovascular disease, cancer, type 2 diabetes, osteoporosis and chronic obstructive pulmonary disease (COPD) (American College of Sports Medicine, 2017; Beck et al., 2017; Fragala et al., 2019; Hayes et al., 2019; Hordern et al., 2012; Izquierdo et al., 2021; Morris et al., 2021; Selig et al., 2010; Sharman et al., 2019). Notably, many patients with these diseases are also characterized by traits consistent with sarcopenia, with significant impairments in the capacity to perform ADLs and poorer quality of life (Bekfani et al., 2016; Leenders et al., 2013). According to the American College of Sports Medicine (ACSM), RT should be a component of every exercise program for healthy adults, and those with clinical conditions (American College of Sports Medicine, 2017). Extensive evidence on the benefits of RT in healthy older adults and those with chronic disease is well defined, but the question remains: "*Does RT prescription change if a patient is diagnosed with sarcopenia, defined by any or different definitions*?" The answer is probably not.

4. Resistance training guidelines: show me the differences!?

The general principle of RT prescription is that the exercise programs should be progressed and individualized to each person (American College of Sports Medicine, 2009). Many organizations have and continue to release independent RT guidelines for older adults and clinical populations (American College of Sports Medicine, 2017; Izquierdo et al., 2021). Regarding sarcopenia, while it is independently recognized as a disease, it commonly unveils as a consequence of many other chronic diseases, even despite substantial differences in pathophysiology, progression and symptoms (Collamati et al., 2016; Mesinovic et al., 2019; Pacifico et al., 2020). The pharmacological treatment of each disease vary in many ways, but the RT recommendations are similar. While it is not possible to provide an overview of all available guidelines from various organizations, herein we provide a proof of concept to demonstrate the similarities in RT guidelines across the healthy and disease continuum, using best practice clinical exercise guidelines from ACSM and recommendations for healthy individuals from both ACSM and the International Conference on Frailty and Sarcopenia Research (ICFSR) consensus (Table 1) (American College of Sports Medicine, 2017; Izquierdo et al., 2021). We have focused, in particular, on diseases commonly observed in conjunction with sarcopenia.

When observing Table 1, there are noticeable similarities existing regarding resistance exercise prescription guidelines, irrespective of disease status. Regarding exercise intensity, some evidence suggests that similar increases in muscle strength have been observed when using an intensity of either moderate or heavy loads (between 40% and 90% of 1-repetition maximum, 1RM) once total volume is accounted for and if

Table 1
Internationally recognized exercise prescription guidelines for older adults and common clinical populations that share characteristics of sarcopenia.

Association (year)	Population	Frequency (days per week)	Exercise prescription for resistance exercise		
			Sets	Reps	Intensity
ICFSR Consensus (2021) (Izquierdo et al., 2021)	Older adults	2–3	1–2	8–12	50–80% 1RM
ACSM (2017) (American College of Sports Medicine, 2017)	Older adults	2–3	1	10–15	40–50% 1RM
ACSM (2017) (American College of Sports Medicine, 2017)	Healthy	2–3	2–4	8–12	60–70% 1RM
ACSM (2017) (American College of Sports Medicine, 2017)	Obese	2–3	2–4	8–12	60–70% 1RM
ACSM (2017) (American College of Sports Medicine, 2017)	Type two diabetes	2	1–3	10–15	Moderate (50–69% 1RM) to vigorous (70–85% 1RM).
ACSM (2017) (American College of Sports Medicine, 2017)	Cardiovascular disease	2–3	1–3	10–15	40–60% 1RM, BORG RPE 11–13
ACSM (2017) (American College of Sports Medicine, 2017)	Chronic heart failure	1–2	2	10–15	40–70% 1RM
ACSM (2017) (American College of Sports Medicine, 2017)	Chronic kidney disease	2–3	1	8–12	65–75% 1RM (1RM estimated from 3RM)
ACSM (2017) (American College of Sports Medicine, 2017)	Peripheral arterial disease	2	1	8–12	60–80% 1RM
ACSM (2017) (American College of Sports Medicine, 2017)	Dyslipidaemia	2–3	2–3	8–12	Moderate (50% 1RM) to vigorous (75–80% 1RM), < 50% 1RM to improve endurance
ACSM (2017) (American College of Sports Medicine, 2017)	Hypertension	2–3	2–4	8–12	60–80% 1RM *older adults 40–50% 1RM
ACSM (2017) (American College of Sports	Arthritis	2–3	2–4	8–12	60–80% 1RM *lower intensity for

Table 1 (*continued*)

Association (year)	Population	Frequency (days per week)	Exercise prescription for resistance exercise		
			Sets	Reps	Intensity
Medicine, 2017)					untrained (50–60%)

lower loads are carried out to fatigue (da Silva et al., 2018; Morton et al., 2016). In addition, comparable improvements in strength can be seen in older adults who performed two versus three days per week of RT (Ralston et al., 2018; Stec et al., 2017).

Given that a surprisingly low number of older adults currently meet exercise guidelines (Bennie et al., 2019), researchers and clinicians should focus on how to engage individuals in RT that is enjoyable in order to increase adherence for long lasting benefits. The RT guidelines presented could be summarized by suggesting to complete structured exercise at least two to three days per week. Additionally, to combine whole body movements including upper and lower body exercises, of two to four sets each, and using a rep range that can be completed using a moderate to heavy intensity load until fatigue. Importantly, individualization of each component should occur regardless of disease state. This broader summary indicates that the general recommendations for RT in conditions with traits of sarcopenia do not differ substantially. The lack of variation in these guidelines also suggests that, in terms of RT guidelines, a specific disease diagnosis does not result in a major different RT recommendation.

However, it should be acknowledged that there are different approaches to RT. One of which is power training, a specific type of RT where muscle contractions are performed at high velocity. This type of exercise improves muscle power and has been associated with improved capacity to perform ADLs in older adults (Lopes et al., 2016; Ramírez-Campillo et al., 2014; Reid and Fielding, 2012), in those with mobility limitations (Reid et al., 2015), and even in those that are frail (Cadore et al., 2014a; Coelho-Júnior and Uchida, 2021). However, power training is yet to be incorporated into RT guidelines and as such, expertize from the exercise professional and caution should be considered if it is to be used as part of a RT program. Specifically, when prescribing RT to older adults (with, or without sarcopenia traits), guidelines should be adopted as a guide to clinical practice in-conjunction with an in-depth knowledge of patient' conditions and treatments. This approach will assist with the delivery of an optimal exercise program that can be performed safely by the individual.

Indeed, the definition of sarcopenia itself may also yield a different understanding of what is being treated i.e. increasing muscle mass or/and increasing muscle strength (i.e. handgrip strength) or/and improving physical performance (reducing time to complete the timed up and go). Furthermore, the prescription of any treatment, even exercise, requires an assessment of both risk and benefit for the individual. The risk associated with RT is typically minimal if it is prescribed within the guidelines of ACSM (American College of Sports Medicine, 2017) and it is usually only associated with a delayed onset of muscle soreness (DOMs)(Cheung et al., 2003). DOMs is a common experience following RT and can be experienced by individuals of all fitness levels following unaccustomed physical activity. It is typically characterized by muscle soreness and discomfort that increases with intensity within the first 24 h following exercise, and usually subsiding within a few days (Armstrong, 1984; Cheung et al., 2003).

The benefits of RT in older adults and clinical populations with characteristics of sarcopenia go beyond the skeletal muscle level (i.e. improved strength) and includes improved capacity to perform ADLs, increased cognitive function (Cassilhas et al., 2007; Martín Del Campo Cervantes et al., 2019) and improved quality of life (Giuliano et al., 2017; Levinger et al., 2007). RT also reduces cardiometabolic risk factors (Balducci et al., 2012; Hsieh et al., 2018). For older adults who are frail, living in nursing homes or institutionalized, and often

characterized by multimorbidity, the evidence for beneficial effects of RT on such outcomes is conflicting, likely due to large heterogeneity of the population as well as the definition for frailty used (Chin et al., 2008). The degree of frailty may also be critical in the effectiveness of an exercise program (Chin et al., 2008). However, benefits including improved functional outcomes (Cadore et al., 2014b; Fiatarone et al., 1990; Hassan et al., 2016; Martín Del Campo Cervantes et al., 2019) and quality of life (Cadore et al., 2014b) have been reported in this population (Cadore and Izquierdo, 2015; Cadore et al., 2013). Indeed, there will be some instances whereby RT may not be suitable in particular populations due to very low function level or safety considerations. In that scenario, exercise prescription should be modified, and adapted to the physical function level of the individual taking into account comorbidities, and risk/benefit to participation.

5. Other considerations

This review focuses on RT as a treatment for sarcopenia. However, it is important to acknowledge that other lifestyle interventions may assist in muscle mass and strength preservation during ageing. This includes a balanced approach to the diet including a variety of nutritious foods from the five food groups: vegetables, fruits, grains, proteins (i.e. lean meat, fish, nuts and legumes) and dairy (milk, yoghurt etc.) (Brownie et al., 2015). Prospective studies have demonstrated that when dietary protein intake is low it is linked to functional decline in older adults (Bradlee et al., 2017; Mendonça et al., 2019; Mustafa et al., 2018b; Yuan et al., 2021). Some evidence also demonstrates that adequate protein intake (>1 g/kg/day) can reduce the rate of decline in hand grip strength and mobility (McLean et al., 2016; Mustafa et al., 2018a). However, supplementation with protein above recommended levels in older men > 65 years who were functionally limited, had no effect on muscle mass, strength or power (Bhasin et al., 2018). Recommendations advise that older adults (> 65 years) have higher daily protein requirements than younger adults to maintain/regain lean mass and function, and, these requirements increase for those that exercise, and are even higher again for those with acute or chronic illness (Bauer et al., 2013).

Some evidence suggests a possible additive effect on muscle strength in older adults when combining protein intake with RT (Cermak et al., 2012; Dickinson et al., 2013; Tieland et al., 2012) or a physically active lifestyle (Bradlee et al., 2017). However, others do not support this link (de Carvalho Bastone et al., 2020; Mertz et al., 2021; Roschel et al., 2021), particularly if dietary protein is already adequate prior to beginning RT (Kirk et al., 2019b; Labata-Lezaun et al., 2020). For detailed evidence on nutritional interventions for maintaining muscle mass and strength into old age please see a recent review by Cruz-Jentoft (Cruz-Jentoft et al., 2020).

6. Where from here: use it or lose it

The reduction in muscle mass, strength and function is part of the ageing process, and it creates a challenge to individuals and health care systems globally. Whether an individual is diagnosed as “sarcopenic” or not has no effect on the way RT is prescribed based on current recommended guidelines. As such, the current focus on refining sarcopenia definitions, where five (or more) already exist, may in fact do more harm than good as it may add confusion in the identification of sarcopenia. Moreover, it is plausible that researchers and clinicians will handpick the definition most relevant to their needs. A large body of research demonstrates that RT and a healthy diet including adequate dietary protein and energy intake, remains the best approach in our efforts to prevent and manage loss of muscle mass, strength and physical function. It also provides broader health benefits such as risk reduction for cardiovascular and metabolic disease. From a clinical perspective, regular exercise is recognized as a cornerstone for public health, and yet, despite known health benefits, a large percentage of adults do not meet recommended guidelines (Merom et al., 2012; National Center for Health Statistics, 2015). Moreover, an abundance of studies demonstrate the importance of targeted resistance exercise, yet self-initiated participation levels are low (Burton et al., 2017; Merom et al., 2012). Future focus for research should be aimed at understanding how to increase engagement and long-term adherence to exercise, importantly RT, to prevent functional decline and morbidity.

Funding

The research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

CRediT authorship contribution statement

CS and IL contributed to all aspects of this manuscript including concept and drafting. MNW and MS contributed to relevant areas of their expertize. All authors reviewed and approved the final manuscript.

Acknowledgments

MS is supported by a Royal Perth Hospital Research Foundation Career Advancement Fellowship (CAF 130/2020) and an Emerging Leader Fellowship from the Western Australian Future Health Research and Innovation Fund (Department of Health, Western Australia).

Conflict of interest

the authors have none to declare.

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Appendix 2 Supplementary table 1. Correlative link between total OC and glucose control indices

Supplementary table 1. Correlative link between total OC and glucose control indices

Study	**Population** Sample size (n) Mean age (yrs)	**Assay** T: time of sampling C: fasting/non-fasting	**Main outcome** Mean±SD (unless stated)
Achemlal, (564) Cross-sectional	**Males with poorly controlled T2D** n= 75 (35 T2D/35 CON) T2D: 54 yrs; CON: 53 yrs	ECLIA, Roche Diagnostics T: a.m.; C: N/R	**↓ tOC T2D vs CON** 15.3±4.1 vs 18.3±5.3 ng/mL
Aoki, (565) Cross-sectional	**Subjects with NGT, IFG, IGT and T2D** **n= 55** (45 M/ 10 F) (NGT 39/PDM 11/T2D 5) M 47 yrs; F 56 yrs	Biomedical Technologies Inc T: a.m.; C: fasting	**↑ tOC T2D vs NGT** 6.2±1.9 vs 4.1±1.3 ng/mL
Bae, (566) Cross-sectional	**Community based men and postmenopausal women with and without MetS** **n= 567** (198 M/ 369 PoM) M: 57 yrs; F: 57 yrs	ECLIA, Roche Diagnostics T: a.m.; C: fasting	**↓ tOC in PoM women with MetS vs non-MetS** 18.9±7.7 vs 22.5±7.3 ng/mL ***ns** in men
Bao (567), Cross-sectional	**181 men who underwent coronary angiography with and without MetS** Non-MetS 76/MetS 105 65 yrs	ECLIA, Roche Diagnostics T: a.m.; C: fasting	**↓ tOC MetS vs non-MetS** 4.5±1.9 vs 4.9±2.4 ng/mL

Study	Population Sample size (n) Mean age (yrs)	Assay T: time of sampling C: fasting/non-fasting	Main outcome Mean±SD (unless stated)
Bao, (414)	**Patients with T2D** n= 59 (29 M/ 10 PreM/20 PoM) 55 yrs	ECLIA, Roche Diagnostics T: N/R; C: fasting	tOC ns males vs females 14.7 ± 4.5 vs 15.61 ± 6.03 ng ⁄mL
Buday, (568) Cross sectional	**Adults exhibiting changes in glucose tolerance** n= 290 (135 F/155 M) F: 47 NGT/GI 89 M: NGT 72/GI 83 F: NGT: 47 yrs; GI: 51 yrs M: NGT 34 yrs; GI 48 yrs	elecys 2010 immunohistochemical automat Roche Diagnostic kits, Germany T: N/R; C: fasting	ns between NGT and GI Women 18.9±7.5 vs 18.1±7.6 ng/mL Men 19.9±8.3 vs 19.1±9.2 ng/mL
Cakatay, (569) Case-control	**Adults with T2D and healthy age-matched CON** n= 70 35 T2D (18 F/17M) 35 CON (20 F/15M) T2D F 56 yrs; M 52 yrs CON F 54 yrs; M 49 yrs	RIA, Diagnostic Systems Laboratories T: a.m.; fasting	**↓ tOC T2D vs CON** F: 0.8±0.1 vs 2.4±0.2 mmol/l M: 0.9±0.1 vs 2.1±0.4 mmol/l Mean±SEM

Study	Population Sample size (n) Mean age (yrs)	Assay T: time of sampling C: fasting/non-fasting	Main outcome Mean±SD (unless stated)
Chen. (346) Cross-sectional	**Middle aged and elderly adults** n= 1729 (783 M/946 F) M 61 yrs, F 69 yrs	RIA T: N/R; C: fasting	**↓ tOC MetS vs non-MetS** 4.5±1.9 vs. 4.9±2.4 ng/ml, no significant difference in PoM with and without MetS 5.3±2.9 vs. 5.4±2.9 ng/ml
Chouodray, (365) Cross sectional	**Pre- and post-menopausal women with T2D and age and BMI matched controls** **n= 98 T2D** (51 PreM/ 47 PoM) n= 102 controls (53 PreM/49 PoM) T2D 50 yrs; CON: 50 yrs *PreM and PoM combined	ELISA (Quidel Corporation) T: a.m.; C: fasting	**↓ tOC T2D vs controls (PreM and PoM combined)** 4.2±1.9 vs 9.7±3.2 ng/mL
Confafreux, (381) Cross sectional	**Elderly men** n=798 65 yrs	two-site IRMA (IRMA, ELSA-OSTEO; CIS Bio International) T: N/R; C: fasting	**↓ tOC MetS vs non-MetS** 18.4±6.9 vs 19.5±6.7ng/mL **↓ tOC in in those with elevated BGL vs normal BGL** 18.6±6.6 vs 20.1±6.9 ng/mL

Study	**Population** Sample size (n) Mean age (yrs)	**Assay** T: time of sampling C: fasting/non-fasting	**Main outcome** Mean±SD (unless stated)
Dalgard, (388) Prospective	**healthy, non-diabetic (at baseline, 1997–2000) adult mono- and dizygotic twins** n= 1071 (574 F/497 M) 38 yrs	CLIA, iSYS, Immunodiagnostic Systems Ltd. T: N/R; C: fasting	Baseline: **tOC ↑ M vs F** 24.4(18.6-33.2) vs 22.3 (17.4-29.9) ng/mL Median (25-75 percentiles) *data not presented for those with and without T2D tOC negatively associated with the prevalence of T2D 0.9 (0.9–0.9) (odds ratio [OR], 95% confidence interval
Daniele, (343)	**Individuals with NGT and IGT (pre-diabetics)** n= 122 (43 NGT/ 122 IGT) 45 yrs	human-specific Milliplex map kit T: a.m.; C: fasting	tOC ns between NGT and IGR **divided according to glucose tolerance status, ↓ tOC in combined IFG-IGT and isolated IGT subjects vs IFG and NGT** 5.4±0.2 and 7.2±0.3 vs. 8.0±0.5 and 8.3±0.3 ng/mL Mean±SEM

Study	Population Sample size (n) Mean age (yrs)	Assay T: time of sampling C: fasting/non-fasting	Main outcome Mean±SD (unless stated)
Dobnig, (344) Prospective cohort	**Elderly females including T2D and CON** n= 1664 (583 T2DM/1081 CON) T2D 83 yrs; CON 84 yrs	intact OC 1-49 and large N-MID fragments 1-43 elecys N-MID osteocalcin T: N/R; C: non-fasting	**↓ tOC T2D vs CON** 33.9±20.8 vs 40.0±21.3 ng/mL
Dou, (570) Cross sectional	**Subjects with and without NAFLD men** n= 1558 (Non-NAFLD 1109/NAFLD 449) All 54 yrs Non-NAFLD 54 yrs; NAFLD 53 yrs	ECLIA Roche Diagnostics T: N/R; C: fasting	**↓ tOC in NAFLD v non-NAFLD** 16.2±4.9 vs 17.1±5.4 ng/mL
Garcia-Martin, (345) Cross-sectional	**Postmenopausal women** n= 54 56 yrs	ECLIA, Roche Diagnostics T: N/R; C: fasting	**↓ tOC in IFG vs NGT** 10.7 ± 6.1 vs 17.3±7.4 ng/mL
Gennari, (571) Cross-sectional	**T2D, T1DM and age, sex matched controls** 40 T2D/43 T1DM/83 CON T2D 63 yrs; T1D 44 yrs CON young- 35 yrs; CON old 63 yrs	intact osteocalcin, DiaSorin Diagnostics T: a.m.; C: fasting	ns between groups

Study	Population Sample size (n) Mean age (yrs)	Assay T: time of sampling C: fasting/non-fasting	Main outcome Mean±SD (unless stated)
Hwang, (410) Cross-sectional	**adults** **n= 425** (NGT 23/pre-T2D 150/ T2D 252) 53 yrs	IRMA, Osteo-RIA CT Kit, Cis Bio International T: a.m.; C: fasting	**tOC different between groups** (NGT, Pre-DM, T2D) *data not available **↓ tOC T2D vs Pre-DM** 15.3 ±6.8 v 19.1±8.9 ng/mL
Hwang, (352) Retrospective	**nondiabetic men** n= 1229 47 yrs	ELISA, Metra TM Osteocalcin (Quidel, Santa Clara, CA) T: a.m,.; C; fasting	**tOC tertiles** T1: 0.83±0.20 mmol/L T2 1.28±0.11 mmol/L T3 1.83±0.32 mmol/L HOMA-IR varied inversely with tOC tertiles. ns BGL, HbA1c Ns development of T2D across tOC tertiles
Im, (334) Cross-sectional	**Postmenopausal women** n= 339 (259 NG/40 IFG/31 T2D) 57 yrs	ECLIA, Roche T: N/R; C: fasting	**↓ tOC T2D vs NG and IFG** 17.5±6.4 vs 22.2±9.4 and 21.1±8.2 ng/mL

Study	Population Sample size (n) Mean age (yrs)	Assay T: time of sampling C: fasting/non-fasting	Main outcome Mean±SD (unless stated)
Kanazawa, (401) Cross-sectional	**PoM and men with T2D (untreated for T2D or osteoporosis) and PoM and men with OGTT data** 101 PoM/ 152 M with T2D 18 PoM / 2 M with OGTT PoM 62 yrs; M 56 yrs	IRMA [125I]-bone gla protein (BGP) as a competitive radioligand, and bound radioactivity was measured using a gamma counter T: N/R; C: fasting	Post OGTT examinations by tertiles based on tOC levels: PoM: **lowest tOC tertile showed hyperglycemia and hyperinsulinemia vs highest tertile** **Men in lowest TOC tertile also exhibited hyperinsulinemia** *levels N/R
Kim, (386) Cross-sectional	**Obese men** n= 86 (15 normal/71 obese/overweight) normal weight 41 yrs Obese/overweight 38 yrs	ECLIA; Roche T: N/R; C: fasting	tOC not different between normal weight and obese/overweight group 14.1±5.2 vs 14.0±4.4 ng/mL **↓ tOC in obese and overweight subjects with visceral obesity** 12.7±3.2 vs 18.6±4.9 ng/mL

Study	Population Sample size (n) Mean age (yrs)	Assay T: time of sampling C: fasting/non-fasting	Main outcome Mean±SD (unless stated)
Kim, (572) Cross sectional	**PreM and PoM women** Unmatched women n= 617 (301 PreM /316 PoM) Matched women n= 122 (61 PreM/61 PoM) Unmatched women PreM 42 yrs; PoM 56 yrs Matched women PreM 47 yrs; PoM 48 yrs	ECLIA, Roche T: N/R; C: fasting	**tOC levels associated with fasting insulin and HOMA-IR in postmenopausal women only** tOC ns between groups *levels not reported
Kindblom, (333) Cross-sectional	**Non-diabetic and diabetic elderly men** n= 1010 (857 non-T2D/153 T2D) 75 yrs	monoclonal antibodies against human OC, 1-43 and 1-49, ECLIA, elecsys N-MID T: a.m.; C: fasting	**↓ tOC T2D vs nondiabetic** 21.7±8.2 vs 27.6±13.8 ug/L
Liao, (384) Cross sectional	**Adult men** n= 2400 (MetS 219 /non-MetS 2181) Non-Mets: 37 yrs; MetS 42 yrs	ECLIA, Roche Diagnostics T: a.m.; C: fasting	**↓ tOC in MetS vs non-MetS** 20.0±1.3 vs 24.0±1.4 ng/mL
Liatis, (394) Prospective	**Adults at high risk of T2D** n= 307 54 yrs	N-Mid Osteocalcin ELISA; Immunodiagnostic Systems Ltd T: N/R; C: fasting	**↓ tOC in those with IFG and/or IGT vs NGT** 6.0±3.1 vs. 7.3±4.0 ng/mL

Study	Population Sample size (n) Mean age (yrs)	Assay T: time of sampling C: fasting/non-fasting	Main outcome Mean±SD (unless stated)
Lerchbaum, (380) Cross sectional	**Adult men and women** **n= 2671** (1449 M/ 1222 F) M 55 yrs; F 54 yrs Median	IDS-iSYS MultiDiscipline Automated Analyser (Immunodiagnostic Systems Limited) T: 8:00am to 8:00pm; C: non fasting	**Higher tOC lower odds of MetS and T2D** Men: 1SD ↑tOC of 7.0 ng/mL OR of MetS = 0.8 (0.7-0.9) Women: 1SD ↑tOC of 9.6 ng/mL OR of MetS = 0.8 (0.6-0.9) Men: 1 SD ↑ntOC of 7.0 ng/mL OR of T2D 0.6 (0.5-0.7) Women: For 1 SD ↑ tOC of 9.6 ng/mL OR of T2D 0.6 (0.5-0.8) OR (95% CI)
Lopes, (573) Cross sectional	**Post-menopausal women with osteopenia/osteoporosis with and without T2D** n= 43 (20 CON/20 T2D) T2D: 59 yrs; CON: 58 yrs	ELISA (DIAsource ImmunoAssays) T: a.m.; C: fasting	**↓ tOC T2D vs controls** 10.2±5.4 vs 14.8±5.3 ng/mL

Study	Population Sample size (n) Mean age (yrs)	Assay T: time of sampling C: fasting/non-fasting	Main outcome Mean±SD (unless stated)
Ma, (574) Cross sectional	**Community based study of middle aged and elderly men with chronic diseases** **n= 1077** (T2D and pre-diabetes 638/ HGT 638/ IGR 302/ NDD: 137) *NDD, new diagnosed diabetes 61 yrs	ECLIA *analyser/manufacturer N/R T: N/R; C: fasting	tOC according to glucose tolerance status **↓ tOC in IGR and NDD than NGT** NGT 19.2 (18.6- 19.8) vs IGR 17.7 (16.8-18.5) vs NDD 17.4(16.1-18.7) ng/mL Median (IQR) ***significantly different among groups**
Movahed, (358) Cross-sectional	**Postmenopausal women** n= 382 59 yrs	N-MID Osteocalcin ELISA (Nordic Bioscience Diagnostics A/S) T: N/R; C: fasting	**below median tOC (lower tOC) vs above median tOC (higher tOC) had higher FBG** 8.2 ±1.1 vs 14.1±1.4 ng/mL ns between MetS vs non-MetS
Oosterwerff, (383) Cross sectional	**Community-dwelling elderly subjects** **n= 1284** (629 M/ 655 W) (MetS: 476/Non-MetS 808) MetS: 75 yrs Non-MetS 75 yrs	IRMA (Biosource Diagnostics) T: a.m.; C: non fasting	**↓ tOC in MetS vs non-MetS** 1.8(1.3-2.4) vs 2.1(1.6-2.8) mmol/L Median(IQR)

Study	Population Sample size (n) Mean age (yrs)	Assay T: time of sampling C: fasting/non-fasting	Main outcome Mean±SD (unless stated)
Oz, (367) Cross-sectional	**T2D and non-T2D** n= 100 52 T2DM (37 F/15 M) 48 Non-T2DM (34 F /14 M) 41-64 yrs (range)	microenzyme-linked immunosorbent assay, micro-ELISA, Tecan T: N/R; C:fasting	**↓ tOC T2D vs CON** 8.1±5.7 vs 15.8±8.24 ng/mL
Papastefanou, (575) Case-control	**Pregnant women** n= 134 (40 GDM/ 94 controls) GDM 33 yrs; controls 30 yrs	N-MID OC, ECLIA; Roche Diagnostics T: 9:00 am to 18:00pm; C: not fasted	**GDM group, first trimester higher tOC vs control** 8.8±2.6 vs 7.3± 3.0 ng/mL
Pittas, (338) Prospective cohort	**Healthy and elderly** n= 380 71 yrs	two site-IRMA, Nichols Institute T: a.m.; C: fasting	Divided into tertiles by tOC **Q3 (Highest tOC) mean FPG was 97.1 *vs.* Q1 (lowest tOC) 104.8 mg/dl**
Sarkar, (366) Cross sectional	**Men with T2D and age and BMI matched controls** n= 112 (56 T2D/56 controls) 52 yrs	ELISA (Quidel Corporation) T: a.m.; C: fasting	**↓ tOC T2D vs CON** 3.6±1.6 vs 7.9±2.5 ng/mL
Shu, (576) Cross-sectional	**Post-menopausal with and without T2D** n=50 (50 T2D/50 CON) T2D 63 yrs; CON 60 yrs	ELISA N-mid Osteocalcin (IDS Ltd.) T & C: N/R	**↓ tOC T2D vs CON** 4.5±2 vs 6.2±2 nmol/L

Study	Population Sample size (n) Mean age (yrs)	Assay T: time of sampling C: fasting/non-fasting	Main outcome Mean±SD (unless stated)
Shu, (364) retrospective	**Middle-aged adults** n= 1870 (1279 men/ 591 women) (1482 NGT/429 IGT/85 IFG) 47 yrs	Elecsys N-MID OC assay, ECLIA Roche T: N/R; C: fasting	*Baseline:* **↓ tOC higher prevalence of IFG, IGT** **Prevalence of MetS lowest in those with highest tOC levels (quartile 4) when cohort was subgrouped by tOC based on quartiles**. Q1 11.2±1.5 vs Q4 24.4±4.9 ng/mL **3-years follow up; those with higher tOC at baseline had lower incidence of diabetes**

Study	Population Sample size (n) Mean age (yrs)	Assay T: time of sampling C: fasting/non-fasting	Main outcome Mean±SD (unless stated)
Szulc, (577) prospective cohort	**MINOS Prospective cohort study of men with osteoporosis** n= 762 MetS 65 yrs, non-MetS 66 yrs	human-specific, two-site IRMA, ELSA-OSTEO; CIS Bio International T: a.m.; C: fasting	**↓ tOC MetS vs non-MetS** 18.3±10.3 vs 19.7±6.9
*follow up study of same cohort **Confavreux**, (381)	**MINOS Prospective cohort study of men with osteoporosis** n= 798 Age: 65 yrs	human-specific, two-site IRMA, ELSA-OSTEO; CIS Bio International T: N/R; C: fasting	**↓ tOC Higher MetS traits vs lower MetS traits** five criteria 15.0±5.1 vs 0–2 criteria 19.5±6.7 ng/mL
Tan, (385) Cross-sectional	**Adult men** **n= 2344** MetS 297/non-MetS 2047 MetS 42 yrs; Non-MetS: 37 yrs	ECLIA, Roche Diagnostics T: N/R; C: fasting	**↓ tOC MetS vs non-MetS** 20.3±1.4 vs 24.1±1.4 ng/mL
Terzi, (578) Prospective, Case-control	**Post-menopausal women with and without MetS** n=230 (63 MetS/167 non-MetS) MetS: 58 yrs; Non-MetS 56 yrs	ECLIA (Roche, Germany) T: a.m.; C : fasting	**↓ tOC MetS vs non-MetS** 17.6±7.0 vs 21.2±5.8 ng/mL

Study	Population Sample size (n) Mean age (yrs)	Assay T: time of sampling C: fasting/non-fasting	Main outcome Mean±SD (unless stated)
Urano, (392) Cross sectional	**PoM women with and without diabetes** n= 1691 (61 T2D/ 1630 controls) 65 yrs	tOC (EIA) T: N/R; C: Non fasting,	**Those with lower baseline tOC <6.1ng/mL had ↑ risk of T2D than those with higher baseline tOC >6.5 ng/mL (over 7.6 ± 6.1 yrs)** **Sig different across quartiles based on tOC levels** Q1 4.2±1.2 vs Q4 12.8±3.2 ng/mL
Winhofer, (579) Case-control study	**Pregnant women with and without gestational diabetes** n= 78 (26 GDM/ 52 NGT) GDM 27 yrs, NGT 28 yrs	ECLIA, Roche Diagnostics, Roche T: N/R; C: fasting	During pregnancy: **↑ tOC in GDM than NGT** 15.6±4 vs 12.6±4.0 ng/mL postpartum ns between groups 36.2±10.2 vs 36.2±13.0 ng/mL
Xu, (580) Cross sectional	**non-T2D with and without first degree relatives with T2D** **N= 1206** (non-FDR 957/ FDR 249) 59 yrs Median	ECLIA, Roche Diagnostics T: N/R; C: fasting	**↓ tOC FDR vs non-FDR** 19.8±5.7 vs 20.7±6.8 ng/mL **Those with NAFLD- those with FDR ↓ tOC than those without FDR** *Levels not reported

Study	Population Sample size (n) Mean age (yrs)	Assay T: time of sampling C: fasting/non-fasting	Main outcome Mean±SD (unless stated)
Yang, (382) Cross sectional	**Community-based post-menopausal women** n= 1789 57 yrs	ECLIA, Roche Diagnostics T: a.m.; C: fasting	**↓ tOC MetS vs non-MetS** 18.5 (15.5-23.5) vs 21.1(16.9-26.3) ng/mL Median (IQR)
Yang, (581) Retrospective	**trauma patients** n= 394 (296 males/98 females) Low HbA1c n = 301 (224 M/77F) High HbA1c n =93 (72 M/21 F) HbA1c-low: 49 yrs HbA1c-high: 52 yrs	N-MID OC automated ECLIA immunoassay (Roche) T: N/R; C: fasting	**low HbA1c group had ↑ tOC vs high HbA1c group** 11.6±5.8 vs 9.1±3.7 ug/L
Yeap, (336) Cross-sectional	**Men > 70 years with and without MetS** n= 2765 (MetS 797/non-MetS 1968) 76 yrs	automated Elecys assay T: a.m. ; C: fasting	**↓ tOC MetS vs no-MetS** 20.1±.4 vs 21.4±.2 ug/L Mean±SEM
Yilmaz, (582) Case- control	**NAFLD and age and sex matched controls** 99 NAFLD/75 CON NAFLD 48 yrs; CON 48 yrs	solid-phase enzyme-amplified sensitivity immunoassay performed on microtiter plates (GenWay hOST-EASIA, GenWay Biotech, Inc.) T: a.m.; C: fasting	**↓ tOC NAFLD vs CON** 2.2 (2.2–2.4 ng/mL) vs 2.3 (2.2–2.4 ng/mL) Median (IQR)

Study	Population Sample size (n) Mean age (yrs)	Assay T: time of sampling C: fasting/non-fasting	Main outcome Mean±SD (unless stated)
Zhang, (583)	**acromegalic patients and sex-, age-, and BMI-matched healthy controls** Acromegalic n=50; controls n=30 48 yrs	ECLIA (Roche Diagnosis, Mannheim, Germany) T: N/R; C: fasting	**↓ tOC acromegalic vs CON** 19.46±6.69 55.45±34.02 ng/mL
Zhang, (584)	**Adult men and women at risk for cardiovascular disease** n= 461 (299 M/162 F) M 62 yrs; F 63 yrs	ECLIA (Elecsys N-MID Osteocalcin Calset; Roche Diagnostics T: a.m.; C: fasting	**↓ tOC in coronanry heart disease vs non-coronoary heart diseae** 12.2 (9.5-15.1) vs (13.6 (10.7-18.0) ng/mL Median (IQR)
Zhou, (585) Cross-sectional	**Postmenopausal women with T2D and age matched non-T2D** (890 T2D/689 CON) 58 yrs	IRMA- ALSA-OSTEO kit. T:N/R; C: fasting	**T2D with low <25 and high BMI >25 have ↓ tOC** vs **controls** **< 25 kg/m2 BMI group** 12.5±0.3 vs 16.9±0.1 **>25 kg/m2 BMI group** 10.2±0.2 vs 12.8±0.1

Study	Population Sample size (n) Mean age (yrs)	Assay T: time of sampling C: fasting/non-fasting	Main outcome Mean±SD (unless stated)
Zhou, (586) Cross-sectional	**Adults with T2D and NGT** n= 500 254 men (128 T2D/126 NGT) 53 yrs 66 PreM (33 T2D/33 NGT) 43 yrs 180 PoM (92 T2D/88 NGT) 62 yrs	ECLIA, Roche Diagnostics T= N/R; C= fasting	**↓ tOC T2D vs NGT** 15.1(10.8-18.3) vs 16.8(11.8-20.6) ng/mL Median(IQR)

Appendix 2 Published version of manuscript: Osteocalcin and its forms across the lifespan in adult men

Bone 130 (2020) 115085

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Full length article

Osteocalcin and its forms across the lifespan in adult men

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ARTICLE INFO

Keywords:
Osteocalcin
Aging
Reference ranges
Bone
Bone turnover

ABSTRACT

Purpose: Osteocalcin (OC), an osteoblast-specific secreted protein expressed by mature osteoblasts, is used in clinical practice and in research as a marker of bone turnover. The carboxylated (cOC) and undercarboxylated (ucOC) forms may have a different biological function but age-specific reference ranges for these components are not established. Given the different physiological roles, development of reference ranges may help to identify people at risk for bone disease.

Methods: Blood was collected in the morning after an overnight fast from 236 adult men (18 to 92 years old) free of diabetes, antiresorptive, warfarin or glucocorticoid use. Serum was analyzed for total osteocalcin (tOC) and the ucOC fraction using the hydroxyapatite binding method. cOC, ucOC/tOC and cOC/tOC ratios were calculated. Reference intervals were established by polynomial quantile regression analysis.

Results: The normal ranges for young men (≤30 years) were: tOC 17.9–56.8 ng/mL, ucOC 7.1–22.0 ng/mL, cOC 8.51–40.3 ng/mL (2.5th to 97.5th quantiles). Aging was associated with a "U" shaped pattern for tOC, cOC and ucOC levels. ucOC/tOC ratio was higher, while cOC/tOC ratio was lower in men of advanced age. Age explained ~31%, while body mass index explained ~4%, of the variance in the ratios.

Conclusions: We have defined normal reference ranges for the OC forms in Australian men and demonstrated that the OC ratios may be better measures, than the absolute values, to identify the age-related changes on OC in men. These ratios may be incorporated into future research and clinical trials, and their associations with prediction of events, such as fracture or diabetes risk, should be determined.

1. Introduction

Osteoporosis affects 1.2 million Australians, with a further 6.3 million affected by osteopenia, with both rates projected to rise in the years to come [1,2]. Six percent of men aged over 50 years have osteoporosis, increasing to 13% in those over 70 years [1,2]. Circulating levels of bone turnover markers (BTMs) are used in research and clinical practice to predict fracture risk [3–5]. Reference intervals and

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https://doi.org/10.1016/j.bone.2019.115085
Received 21 June 2019; Received in revised form 26 September 2019; Accepted 27 September 2019
Available online 14 October 2019
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treatment targets of BTMs for older women, based on premenopausal data, have been extensively characterized, however, only a few studies are available for men [6–8].

Serum total osteocalcin (tOC), an osteoblast-specific secreted protein expressed by mature osteoblasts, is the most abundant, non-collagenous protein found within the bone matrix and is used as a BTM [9,10]. tOC exists in the circulation in two major forms; γ-carboxylated (cOC) and undercarboxylated (ucOC) which lacks γ-carboxylation at one or more sites [11]. cOC is thought to be predominately located in bone, whereas ucOC may participate in glucose metabolism, influencing muscle mass and strength [12–22]. Previous studies indicate circulating tOC is highest in early adulthood, lower in mid-life, and with mixed results shown in older adults [23–31]. Despite the differences in the biological functions of the OC forms, few studies report both forms and tOC levels, or their age-specific distributions. Consequently, normal ranges of OC forms and ratios (ucOC, cOC, ucOC/tOC, cOC/tOC) in men are not quantified.

The aims of the current study were to determine how tOC, ucOC, cOC and the ratios ucOC/tOC and cOC/tOC change with age in adult men and to define normative ranges of the OC forms and OC ratios in this population.

2. Material and methods

2.1. Study population

This is a cross-sectional study representative of men across the adult lifespan, utilizing collected fasted baseline (resting) sera samples of a total of 236 men aged 18 to 92 years. The datasets include data from the following studies: (a) the Health In Men Study (HIMS), a population-based cohort study, comprising of 4248 men aged 70 to 89 years, assessed in 2001-04, who have been followed-up since initial recruitment in 1996 (Perth, Western Australia); From men in HIMS who had previously had tOC and ucOC assessed [12], after excluding men with diabetes, heart disease or osteoporosis, 99 men were randomly selected for the current study; (b) the Nepean Osteoporosis and Frailty (NOF) study, a cross-sectional study of older adults with frailty and other comorbidities (Western Sydney, Australia). A total of 23/76 samples were eligible after exclusions for diseases (i.e. diabetes) and medications known to affect OC and bone metabolism, 23 were eligible [32]; (c) exercise studies at Victoria University investigating bone health, comprising 20 healthy men aged 21 to 70 years [33,34]; (d) the Gene Smart Study, is an ongoing international, multi-center study that is a part of the recently established ATHLOME Consortium [35,36]. To date (April 2019), 94 men have completed the study. At the time of establishing the current study only 74 samples from healthy, young men (aged 18 to 45 years) were collected and were included in the current study; and (e) the Vegetable Intake and Blood Pressure (VIABP) study is a randomised, controlled, cross-over study of 30 non-smoking, non-diabetic participants (20 men, 10 women) with pre-hypertension or untreated grade 1 hypertension, only samples from men, aged 40 to 74 years (first baseline visit), were included [37]. All volunteers signed a consent form for participation in the respective studies.

In total, of the 236 men included in this study, 17 men from the NOF study and 5 men from the VIABP used medications including anti-hypertensives (NOF, n = 9); antiplatelets (NOF, n = 2), nitrates (NOF, n = 2); statins (NOF, n = 7; VIABP, n = 2); ventolin (NOF, n = 2); proton pump inhibitors (NOF, n = 6; VIABP, n = 1); diuretics (NOF, n = 2); non-steroidal anti-inflammatory drugs (NSAIDs) (NOF, n = 2; VIABP, n = 2); opioids (NOF, n = 1); anticholinergic/anti-muscarinic (NOF, n = 1) and vitamin D/calcium (NOF, n = 2).

2.2. Quantification of serum osteocalcin (tOC) and undercarboxylated osteocalcin (ucOC)

The stored sera samples were selected on the following criteria: a) samples collected in the morning following an overnight fast (to minimize circadian variation); b) samples were analyzed at the same laboratory, by the same technician and following the same methodology; and c) all samples were collected as serum and kept in aliquots in long term storage at -80C until assayed and no freeze-thaw cycles previously reported.

Frozen serum samples from each clinical trial were obtained from long-term storage and analyzed using identical technique and equipment, and performed by the same technician. Serum tOC was measured using an automated immunoassay (Elecsys 170; Roche Diagnostics). Serum ucOC was measured by the same immunoassay after absorption of carboxylated OC on 5 mg/mL hydroxyl-apatite slurry, following the method described by Gundberg et al [11] and Chubb et al [8].

The Elecsys N-MID Osteocalcin assay uses two monoclonal antibodies specifically directed against epitopes on the N-MID fragment and the N-terminal fragment. The assay hence detects the stable N-MID fragment as well as the (still) intact OC. The test is non-dependent on the unstable C-terminal fragment (amino acids 43–49) of the OC molecule and thus ensures constant measurement results under routine conditions in the laboratory. Test Principle (from Roche N-MID Osteocalcin product insert). Sandwich immunoassay – assay duration 18 min. 1st incubation: 20 u L of sample, a biotinylated monoclonal N-MID OC specific antibody and a monoclonal N-MID OC -specific antibody labelled with a ruthenium complex [Tris (2,2′-bipyridyl)ruthenium(II)-complex; $Ru(bpy)_3^{2+}$] react to form a sandwich complex. 2nd incubation: after addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell M. Application of a voltage to the electrode then induces a chemiluminescent emission which is measured by a photomultiplier. Results are determined via a calibration curve which is generated by 2-point calibration and a master curve provided via the reagent barcode. The reagents used for the measurement of OC were the Roche N-mid Osteocalcin (Roche Diagnostics, Mannheim) on the Roche E170 Analyzer (Elecys 170; Roche Diagnostics). This is the same reagent and instrument used in previous studies by Chubb et al [8] and Levinger et al [15,18,38]. The hydroxyapatite used was Calbiochem Hydroxyapatite, Fast Flow catalog # 391,947 as described by Gundberg et al [11] and as used in our previous work [15,18,38].

Using commercial control material (Roche Precivaria controls level 1 and 2), the following inter-assay variability was seen over 16 analytical runs for OC on the Roche E170: N = 16, mean 19.02, SD 0.33, CV 1.71%; N = 16, mean 91.41, SD 3.01, CV 3.29%. Using an OC standard material purchased from Sigma chemicals spiked into OC free serum, the following interassay variability was seen over 7 analytical runs for the tOC and % binding to hydroxyapatite: High concentration N = 7, mean total 193.43, SD 14.70, CV 7.60%; High concentration N = 7, mean % bound 79.17%, SD 2.21, CV 2.80%; Low concentration N = 6, mean total 18.21, SD 1.78, CV 9.78%; Low concentration N = 6, mean % bound 73.94%, SD 4.62, CV 6.25%. This % binding was similar to that seen in previous studies [39–41].

2.3. Statistical analysis

All statistical analyses were performed using R version 1.1.453 [42]. We initially intended to use quantile regression as previously published [43] to generate 95% reference ranges for the bone markers and ratios within the cohort, but we noted that age strongly modified the reference ranges. Therefore, instead of using arbitrary age cut-offs and splitting our cohort into smaller age groups, we performed polynomial regression of degree 2 for tOC, cOC and ucOC, and simple regression for the ratios cOC/tOC and ucOC/tOC. We generated 95% reference ranges for each of the bone markers as a continuous function of age, with the predict() function. The 95% reference ranges are easily readable as red

Table 1
Descriptive characteristics of our cohort of fasted adult men.

	Entire cohort (mean ± SD)
Sample (n)	236
Age (years)	58.14 ± 21.73
BMI (kg/m^2)	26.18 ± 3.83
tOC (ng/mL)	24.78 ± 10.58
cOC (ng/mL)	13.51 ± 7.69
ucOC (ng/mL)	11.26 ± 4.48
ucOC/tOC	0.48 ± 0.12
cOC/tOC	0.52 ± 0.12

All data reported as mean ± SD. BMI, body mass index; tOC, total osteocalcin; ucOC, undercarboxylated osteocalcin; and cOC; carboxylated osteocalcin.

dashed lines on the individual figures. We also added body mass index (BMI) as a covariate to each of the models to determine whether adjustment for BMI was required. Reference ranges for men < 30 years were given as the 2.5th-97.5thquantiles of each bone marker. We also report the 95% CI for the upper and lower limits of the reference ranges. All data are presented as mean ± SD. For all statistical analyses, p values < 0.05 were considered statistically significant.

3. Results

A total of 236 men were included with a mean age of 58.1 ± 21.7 years and BMI of 26.2 ± 3.8 kg/m^2 (Table 1, sample of men per decade of age are presented in Table 2). tOC, ucOC and cOC (Fig. 1A-C) all displayed a "U shaped" relationship across the aging continuum, with lowest levels observed around 55 years of age. Specifically, from 18 to 59 years old, tOC levels diminished in a non-linear fashion from ~42 ng/mL to ~18 ng/mL; tOC levels were higher after 59 years of age in a non-linear fashion, attaining ~24.2 ng/mL at 80 years old. ucOC and cOC levels show similar associations with age. From 18 to 52 years old, ucOC levels diminished from ~14.7 ng/mL to ~8.7 ng/mL; ucOC levels increased after 52 years of age, attaining ~12.6 ng/mL at 80 years old. From 18 to 63 years old, cOC levels diminished from ~27.1 ng/mL to ~8.3 ng/mL; cOC levels were higher after 63 years of age, attaining ~11.6 ng/mL at 80 years old.

Mean and SD for the absolute values of OC forms were calculated to determine the dispersion of individual values between young (< 30 years) and older adults (> 70 years); tOC in young men was lower than older adults (35.31 ± 11.56 versus 22.33 ± 10.17); ucOC was similar in young and older men (12.66 ± 4.29 versus 11.59 ± 4.79) and cOC was higher in younger men compared to older (22.65 ± 8.69 versus 10.74 ± 6.22).

In contrast to the individual forms, there was an incremental rise in ucOC/tOC ratio across age (Fig. 1D) while the cOC/tOC ratio was lower (Fig. 1E). Adjusting for the effect of BMI, on average, men have 0.3 ± 0.03% lower cOC/tOC ratio per decade; conversely, increments of 0.3 ± 0.03% of ucOC/tOC per decade of age were observed. Age explained ~31% of the variance while BMI explained ~4% of the variance in the ratios. BMI was not associated with individual measures of tOC, cOC and ucOC and as such was not adjusted for in those models (Table 3).

Table 2
Total number of men per decade of age.

Age group	Total n
< 20 years	8
20 to 29 years	32
30 to 39 years	28
40 to 49 years	18
50 to 59 years	13
60 to 69 years	16
70 to 79 years	93
80 to 89 years	27
> 90 years	1

The recommended reference ranges (2.5th to 97.5th quantiles) and 95% confidence limits for the lower and upper limits, based on the data of the young (< 30 years old), healthy men are presented in Table 4 for all OC forms and ratios.

4. Discussion

We report that in adult men (a) for all OC forms, aging was associated with a u-shape pattern expressed across the lifespan and, (b) age accounted for ~30% of the inter-individual variability in the ucOC/tOC and cOC/tOC ratios.

Circulating tOC is used in clinical practice as one of the measures to assess bone disease and as a surrogate measure for bone turnover [9,10,44,45]. As reported [23,25,27], we confirm that circulating tOC follows a u-shape pattern across the lifespan with high values in young individuals and in older individuals. This pattern with aging was also observed in the current study for cOC and ucOC. Clinically, this observed pattern limits the capacity of using the absolute concentration of OC forms for risk stratification. For instance, mean circulating tOC concentrations of 30 ng/mL can be observed in a 30 year old and 70 year old man, but in younger men it may indicate modelling and remodelling associated with the consolidation of bone, while in older men it may indicate increased bone remodelling, bone loss and emerging bone fragility [4,9–13,16–22,46].

We observed that ucOC was higher in older adults compared to middle aged adults (Fig. 1), but similar in young men. The higher levels of ucOC in older adults is intriguing, ucOC has been reported to be involved in both glucose and lipid metabolism, and low ucOC is associated with an increased risk for cardiovascular disease and diabetes [12,13,21,22,47,48], even after adjustment for body mass index [13]. As such, one may speculate that older adults will have lower ucOC, compared to middle aged individuals, as age is associated with increased risk for diabetes, this however was not supported by the evidence. Whether the increase in ucOC in older adults is due to a reduction in vitamin K intake or a potential compensatory mechanism to maintain normal circulating glucose levels in older adults is not clear and should be explored in future studies.

In contrast to absolute OC values, OC ratios (cOC/tOC and ucOC/tOC) may be more sensitive to the physiological changes associated with aging. In the current study cOC/tOC ratio was significantly lower in advanced age. Others have demonstrated that lower cOC/tOC ratio can predict fracture risk particularly in older men [49]. Taken together, our data indicates that the ratio may be more useful for risk stratification and likely provides a better reflection of disease status including osteoporosis, fracture risk or metabolic diseases. Future research that includes clinical outcomes is required to confirm this.

The reference ranges for tOC have previously been established and are commonly used in clinical practice [25,50]. In the current study, we used data from young, healthy men to estimate the reference range for "optimal" levels as it corresponds to the time where bone mass peaks [51]. Our reported reference range for young, healthy men for tOC was 17.9 to 56.8 ng/mL (2.5th to 97.5th quantiles), which is slightly lower than the clinical standard. We have used fasting and morning sampling times to minimize the effect of diurnal variation and feeding, both of which are reported to effect BTMs [52–54]. This may suggest the references range used in clinical practice appears to be acceptable, however may need to be slightly adjusted.

As described above, assessing tOC limits the capacity to differentiate men based on age and to interpret underlying pathophysiology, therefore better clinical differentiation of this protein and its potential biological effects are needed [22–28,55]. We are not aware of any published reference interval data across the adult lifespan in men for cOC, ucOC or the ratios of cOC/tOC and ucOC/tOC. One study published

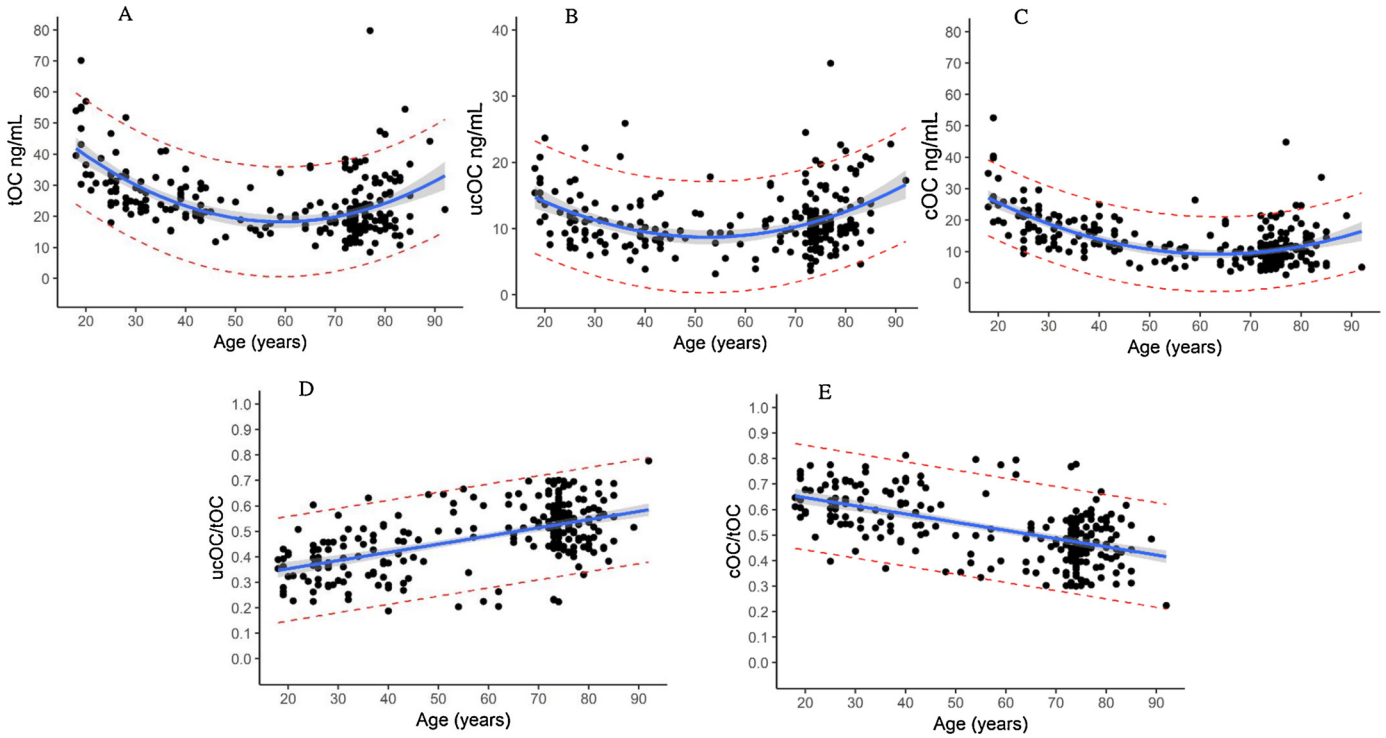

Fig. 1. Relationship between age and circulating levels of tOC, ucOC, cOC and the OC-ratios with confidence and prediction intervals in healthy adult men.

Table 3
β estimates: Regression coefficients for all OC forms and ratios in adult men.

	β estimate Regression coeff. Estimate ± Std. error	p value	Adjusted R^2
tOC (ng/mL)			
Age	−1.6 ± 0.20	**4.20 × 10 -14*****	**0.29**
Age2	0.014 ± 0.0019	**7.73 × 10 -12*****	
cOC (ng/mL)			
Age	−1.11 ± 0.138	**4.30 × 10-14*****	**0.39**
Age2	0.00877 ± 0.00130	**1.11 × 10-10*****	
ucOC (ng/mL)c			
Age	−0.53 ± 0.097	**9.98 × 10 -8*****	**0.11**
Age2	0.0051 ± 0.0091	**7.25 × 10 -8*****	
ucOC/tOC			
BMI	0.00692 ± 0.00173	**8.49 × 10-5*****	**0.35 (adjusted to BMI)**
Age	0.00300 ± 0.000305	**< 2 × 10-16*****	**0.31 (unadjusted)**
cOC/tOC			
BMI	−0.00692 ± 0.00173	**8.49 × 10-5*****	**0.35 (adjusted to BMI)**
Age	−0.00300 ± 0.000305	**< 2 × 10-16*****	**0.31 (unadjusted)**

*** p-value ≤0.01, * p-value ≤0.05. BMI, body mass index; tOC, total osteocalcin; ucOC, undercarboxylated osteocalcin; and cOC; carboxylated osteocalcin.

Table 4
Normal reference ranges and 95% confidence limits for a reference cohort of young, healthy men.

	Reference range (2.5th-97.5th quantiles)	95% CI for the limits of the reference range	
		Lower limit	Upper limit
tOC (ng/mL)	17.85 - 56.78	14.30 – 20.65	54.00 - 70.20
ucOC (ng/mL)	7.07 - 22.03	6.00 - 7.58	17.60 - 23.68
cOC (ng/mL)	8.51 - 40.33	4.28 – 10.75	33.33 - 52.60
ucOC/tOC	0.23 - 0.60	0.22 - 0.26	0.54 - 0.70
cOC/tOC	0.39 - 0.77	0.29 - 0.44	0.74 - 0.78

tOC, total osteocalcin; ucOC, undercarboxylated osteocalcin; and cOC; carboxylated osteocalcin.

data distributions for cOC and cOC/tOC [49], and another published reference intervals for ucOC and ucOC/tOC [8], however both studies were performed in older men > 70 years old. We propose that the ratios of ucOC/tOC and cOC/tOC should be used in both clinical practice, and in future research, as they are potentially more robust measures to a) distinguish the effect of aging on OC forms, and b) better understand the underlying aberrant physiology and biological action of OC in general.

There are some potential limitations of our study. The current study focused on ranges of OC levels in men across the adult lifespan aged 18 to 92 years by combining separate study cohorts with different protocols for inclusion and exclusion criteria's and with different geographical locations which may introduce bias in our results. Whilst this study encompasses a large age range of adult men, there is only a small number of men in the youngest and oldest groups. However, the samples used in the current study were all from men without diseases (i.e. diabetes and osteoporosis) and medications (i.e. bisphosphonates and glucocorticoids) known to effect OC and bone metabolism. We have

used the methods proposed by Gundberg et al. [11] to analyze cOC and ucOC, which is considered as the gold standard. However, different research groups use different methodologies to analyze ucOC and the levels depend on the technical details including antibody, specific surface of the hydroxyapatite, amount of the apatite or ELISA used. As such, the reference ranges calculated in the current study may not reflect the levels analyzed with different techniques. The study is also cross-sectional in nature and does not include clinical measures hence, we cannot ascertain whether these reference ranges are associated with incident or prevalence of disease. In the current study the ucOC/tOC in young men was relatively high. It is not clear why the fraction of ucOC was high and plausible explanations may include a non-specific binding to C-terminal fragments which do not contain GLA or diet with low vitamin K, which is required for OC carboxylation. Lastly, several factors may affect circulating OC levels including vitamin D or K, and although we did include people on vitamin K supplementation, we did not measure it.

The strengths of our study include a full adult age range in men, a population at higher risk of cardiovascular diseases and poorer outcomes after bone fracture [56–58]. Additionally, all samples were collected at the same time of day and in a fasted state. Therefor this is the first step in the validation of reference ranges for all OC forms and ratios in adult men. In addition, all OC analysis was completed by the same technician, in the same laboratory and utilizing the same methodology and assays, minimizing variation due to technical error. The methods used in this study are automated and widely available however, the reference ranges proposed are only valid for the measurement of OC using the same assay, technical aspects and methodology. Measurement of OC may also be different according to countries and, therefore may limit the generalizability of the data. Future studies should explore the effect of aging on OC forms in women as the ranges and pattern of change across the adult lifespan may be different than observed for men.

In summary, we have defined normal reference ranges for the OC forms and demonstrated that the OC ratios may be better measures, than the absolute values, to identify the aging effect on OC in men. These ratios may be incorporated into future research and clinical trials, and their associations with prediction of events, such as fracture or diabetes risk, should be determined.

Authors' roles

Study design and drafting of manuscript: CS, JL, IL. Study conduct and data collection: GD, AAS, SP, TBS, LP, NE, SV, DH, DS, XY, SL, MJ, FM, LB, LF, EB, BBY and IL. Data interpretation and analysis: CS, SV, JL, ES and IL. Review of the manuscript and approval of the final version: CS, JL, IL GD, AAS, SP, TBS, LP, NE, SV, DH, DS, XY, SL, MJ, FM, EB, LB, LF, BBY. IL takes responsibility for the integrity of the data analysis.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institution and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Acknowledgments

None.

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Appendix 3 Published version of manuscript: Higher undercarboxylated to total osteocalcin ratio is associated with reduced physical function and increased 15-year falls-related hospitalizations: The Perth Longitudinal Study of Ageing Women

Smith, C., Lewis, J.R., Sim, M., Lim, W.H., Lim, E.M., Blekkenhorst, L.C., Brennan-Speranza, T.C., Adams, L., Byrnes, E., Duque, G., Levinger, I. and Prince, R.L. (2021), Higher Undercarboxylated to Total Osteocalcin Ratio Is Associated With Reduced Physical Function and Increased 15-Year Falls-Related Hospitalizations: The Perth Longitudinal Study of Aging Women. J Bone Miner Res, 36: 523-530. https://doi.org/10.1002/jbmr.4208

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Appendix 4 Published version of manuscript: The effects of acute exercise on bone turnover markers in middle-aged and older adults: a systematic review

Bone 143 (2021) 115766

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Review Article

The effects of acute exercise on bone turnover markers in middle-aged and older adults: A systematic review

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ARTICLE INFO

Keywords:
Acute exercise
Ageing
Bone turnover markers
Intensity

ABSTRACT

Background: Bone turnover is the cellular machinery responsible for bone integrity and strength and, in the clinical setting, it is assessed using bone turnover markers (BTMs). Acute exercise can induce mechanical stress on bone which is needed for bone remodelling, but to date, there are conflicting results in regards to the effects of varying mechanical stimuli on BTMs.

Objectives: This systematic review examines the effects of acute aerobic, resistance and impact exercises on BTMs in middle and older-aged adults and examines whether the responses are determined by the exercise mode, intensity, age and sex.

Methods: We searched PubMed, SCOPUS, Web of Science and EMBASE up to 22nd April 2020. Eligibility criteria included randomised controlled trials (RCTs) and single-arm studies that included middle-aged (50 to 65 years) and older adults (>65 years) and, a single-bout, acute-exercise (aerobic, resistance, impact) intervention with measurement of BTMs. PROSPERO registration number CRD42020145359.

Results: Thirteen studies were included; 8 in middle-aged ($n = 275$, 212 women/63 men, mean age = 57.9 ± 1.5 years) and 5 in older adults ($n = 93$, 50 women/43 men, mean age = 68.2 ± 2.2 years). Eleven studies included aerobic exercise (AE, 7 middle-aged/4 older adults), and two included resistance exercise (RE, both middle-aged). AE significantly increased C-terminal telopeptide (CTX), alkaline phosphatase (ALP) and bone-ALP in middle-aged and older adults. AE also significantly increased total osteocalcin (tOC) in middle-aged men and Procollagen I Carboxyterminal Propeptide and Cross-Linked Carboxyterminal Telopeptide of Type I Collagen in older women. RE alone decreased ALP in older adults. In middle-aged adults, RE with impact had no effect on tOC or BALP, but significantly decreased CTX. Impact (jumping) exercise alone increased Procollagen Type 1 N Propeptide and tOC in middle-aged women.

Conclusion: Acute exercise is an effective tool to modify BTMs, however, the response appears to be exercise modality-, intensity-, age- and sex-specific. There is further need for higher quality and larger RCTs in this area.

1. Introduction

The skeleton has protective, mechanical and metabolic roles, providing structural support and a site for calcium storage [1–3]. Bone should be strong, to prevent fractures, but light, to enable movement in a gravitational environment [1]. Bone turnover, the cellular machinery

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https://doi.org/10.1016/j.bone.2020.115766
Received 25 August 2020; Received in revised form 8 November 2020; Accepted 18 November 2020
Available online 21 November 2020
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responsible for bone integrity and strength, is a finely balanced process responsive to mechanical loads and hormonal changes [4–6].

Exercise is a non-pharmacological intervention that can improve bone health and reduce the risk of osteoporosis [7–11]. The anabolic effects of exercise on osseous tissues are positively associated with the amount of mechanical strain exerted [12]. In animals, the strain-adaptive remodelling response requires intermittent and dynamic, but not static, loading [13–18]. Additionally, loading periods only need to be very short to stimulate adaptive responses, and that bone formation is threshold-driven and influenced by strain rate, frequency, amplitude and duration of loading [17,19–23]. Altogether, these findings demonstrate that bone requires dynamic (not static) strains (i.e. impact loading) for adaptive responses and, higher physiological rates compared to low rates and applied rapidly, to increase this response [14–16,19,24].

In humans, higher impact activities with rapid rates of loading (i.e. tennis/squash) are more osteogenic compared with lower impact sports (i.e. running/cycling) [25–27]. Mechanical loads, produced by exercise, change local microenvironments of the canalicular networks within the bone framework via dynamic fluid shifts stimulating local osteocytes and ultimately bone turnover [28–30]. Exercise serves varying purposes across the lifespan. In children, exercise is important for optimisation of peak bone mass, whereas, in older adults, exercise serves to maintain/reduce the rate of bone loss [9,10,31]. However, the search for a relationship between exercise and bone mineral density (BMD) demonstrates contradictory findings, some reporting beneficial effects [7,11,32], while others have not [33–35]. Moreover, available human data shows that the magnitude of benefit on bone from exercise is inconsistent, often influenced by safety concerns leading to conservatively prescribed training loads [36–40].

To optimise exercise effects on bone health a better understanding of the metabolic responses of bone tissue to various mechanical stimuli is needed. By convention, BMD is widely used as a measure of bone health to predict fracture risk [41], however, it represents a static bone mineral status and cannot be used to estimate acute bone metabolic changes such as those induced by acute exercise. Therefore, BTMs represent an easy to measure option to assess the dynamic fluctuations in bone turnover (Table 1) [42]. Using BTMs to describe bone metabolic activity comes with complexities, contributing to the lack of consensus in the literature. While these markers are sensitive, they have high biological variability attributed to differences in i.e. blood sampling, study protocols, effects of feeding and circadian rhythm [42–44]. As such, the aims of this systematic review were to 1) examine the effects of acute exercise on BTMs in adults over 50 years of age and to determine if middle-aged and older adults respond differently, and 2) to understand whether these effects were exercise modality-, exercise intensity-, sex- or BTM-specific.

2. Methods

This systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines [45] and was registered in the International Prospective Register of Systematic Reviews (PROSPERO) - CRD42020145359.

Table 1
Markers of bone turnover that have been used in the exercise literature.

Markers of bone resorption	
C-terminal crosslinked telopeptide of type I collagen	CTX, Crosslaps
Cross-linked carboxyterminal telopeptide of type I collagen	ICTP
Sclerostin	SCL
Markers of bone formation	
Alkaline phosphatase (total)	ALP
Alkaline phosphatase (bone specific)	B-ALP
Procollagen I carboxyterminal propeptide	*PICP*
Procollagen type 1 N propeptide	*P1NP*
Osteocalcin	OC

2.1. Inclusion criteria

The inclusion criteria for studies in brief were: (i) randomised controlled (RCT), cross-sectional or single arm trials including quasi-randomised design; (ii) adults ≥50 years of age, middle-aged adults defined as mean age ≥50 to <65 years and older adults defined as mean age ≥65 years; (iii) intervention of interest includes acute bout or single-bout of exercise; and (v) outcome of interest was BTMs (*see* supplementary 1, *PICOS protocol*).

2.2. Data extraction

CS and AT performed the literature search (supplementary 2, *search strategy*) and extracted data from the included studies, IL revised discrepancies. The following data were extracted: (i) characteristics of the participants i.e. sample size, sex, age (years), height (centimetres), weight (kilograms) and body mass index (BMI, height/weight^2); (ii) details of the acute exercise bout (intensity, duration, volume, mode); and (ii) details of outcomes of interest (BTMs) measured at baseline and post-acute exercise.

2.3. Quality assessment: risk of bias and methodological index for non-randomised studies

Risk of bias assessments were independently conducted by CS and AT. RCTs were assessed using the Cochrane Collaborations Risk of Bias 2 (ROB2) tool [46]. We assessed selection bias (random sequence generation, allocation concealment), performance bias (blinding of participant and personnel), detection bias (outcome assessor blinding), attrition bias (handling of incomplete outcome data) and other bias including baseline imbalance on the primary outcome and selective reporting. All other trials not meeting the criteria for a RCT were assessed using the Methodological Index for Non-Randomised Studies (MINORS) scale [47].

3. Results

We identified 3637 articles. After removal of duplicates, 1465 unique titles and abstracts were screened, and 1421 articles were excluded. The full text of 44 articles was reviewed and a further 31 were excluded, leaving 13 articles for inclusion in our qualitative synthesis (Fig. 1). The authors of four studies were contacted for further information [48–51]. One intervention was described in two articles but with different stratification of groups, both articles were included and considered as a single trial [52,53]. Another study had additional analyses published at a later date, both articles were included but considered as a single trial [50,51]. Herein for both of these studies, the first published paper will be referenced.

3.1. Quality assessment

Results of the methodological quality assessments are shown in Table 2 and Fig. 2. Only 3 studies were RCTs [54–56] and assessed using the ROB2 tool. All others were assessed using the MINORs scale. No studies achieved a maximum quality score. Scores ranged on the ROB2 (Fig. 2) and on the MINORs scale (Table 2) from 43.8% to 87.5%. The most common source of likely methodological bias using the ROB2 tool was the randomisation process and deviations from the intended study endpoint. Using the MINORs scoring system, the likely source of methodological bias was the absence of unbiased assessment of the study endpoint ($n = 10$) and prospective calculation of study sample size ($n = 8$).

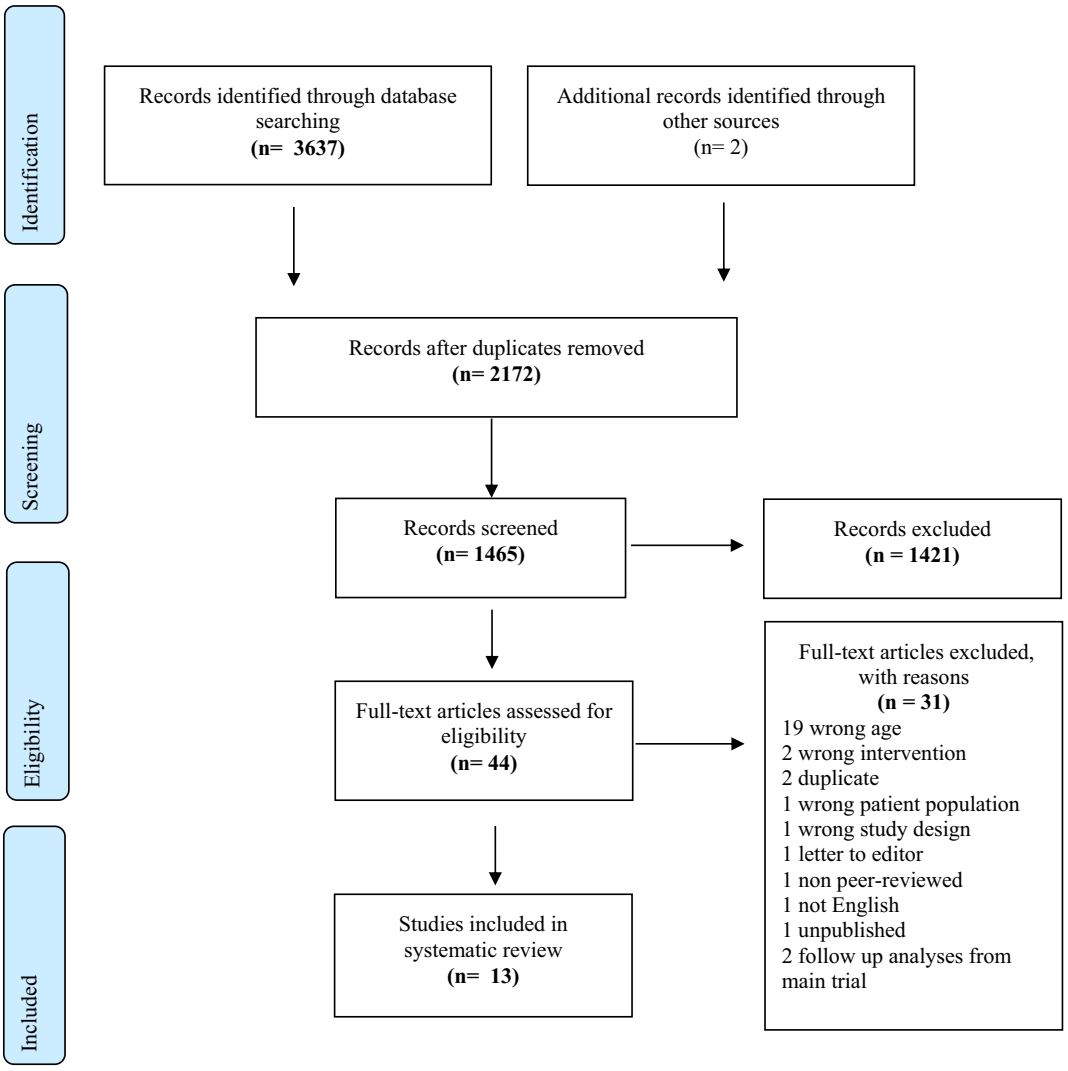

Fig. 1. Identification screening and selection of studies (PRISMA Flow Diagram).

Table 2
Quality rating scale (MINORs).

MINORs Scale (detailed below and scored as: 0, not reported; 1, reported but inadequate; 2, reported and adequate)
Field 9 to 10 only relevant for comparative studies

Author, year	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	Score
1. Kim, et al. (2014)	1	1	0	1	0	2	2	0	n/a	n/a	n/a	n/a	43.8%
2. Levinger, et al. (2011)	2	2	2	2	0	2	2	0	1	2	2	2	79.2%
3. Levinger, et al. (2014)	2	2	2	2	0	2	2	2	n/a	n/a	n/a	n/a	87.5%
4. Maimoun, et al. (2005)	1	2	2	2	0	2	2	0	1	1	2	2	70.8%
5. Rudberg, et al. (2000)	2	1	0	2	0	2	2	0	n/a	n/a	n/a	n/a	56.3%
6. Thorsen, et al. (1995)	2	1	1	2	0	2	2	0	n/a	n/a	n/a	n/a	62.5%
7. Thorsen, et al. (1996)	2	1	1	2	0	2	2	0	n/a	n/a	n/a	n/a	62.5%
8. Aly, et al. (2017)	1	1	2	2	0	2	2	0	n/a	n/a	n/a	n/a	62.5%
9. Wherry, et al. (2019)	2	2	2	2	0	2	2	2	1	2	2	1	83.3%
10. Zerath, et al. (1997)	2	1	2	2	0	2	2	0	n/a	n/a	n/a	n/a	68.8%

MINORs Scale assessed as per; 1. A clearly stated aim; 2. Inclusion of consecutive patients; 3. Prospective data collection; 4. Endpoints appropriate to study aim; 5. unbiased assessment of study endpoint; 6. follow up period appropriate to the aim; 7. loss to follow up <5%; 8. Prospective calculation of study size; 9. adequate control group; 10. contemporary groups; 11. Baseline equivalence of groups and 12. Adequate statistical analysis.

3.2. Study population and study design

Descriptive characteristics and study outcomes of included studies are described in Table 3. Two studies included adults with osteoporosis (untreated) [55,57], five studies excluded individuals with osteoporosis/conditions affecting bone metabolism [49,50,53,54,58] and one study included adults with osteopenia [48]. Four studies did not state whether they excluded participants with osteoporosis [56,59–61]. Five studies excluded individuals taking medications/supplements that effect bone metabolism [48,50,53,54,58], one stated except for calcium

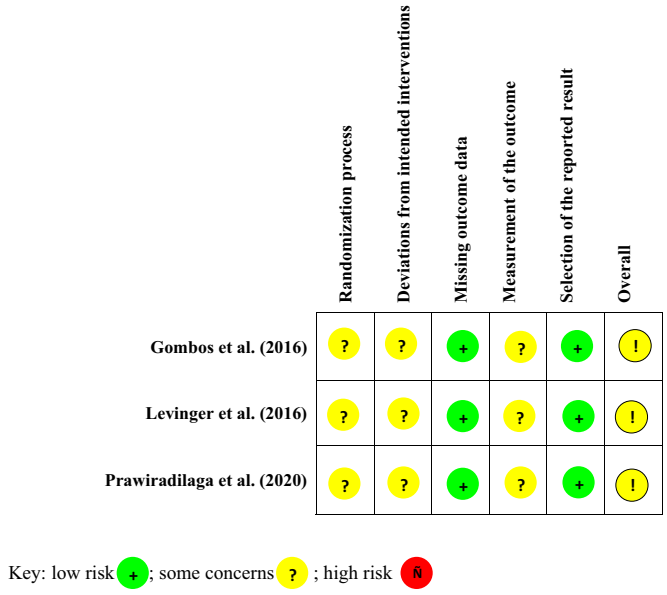

Fig. 2. Risk of bias ratings.

and vitamin D [55], four studies included participants not taking medications [57,60–62] and three studies did not refer to medication use [49,56,59].

Of the thirteen studies included, eight were in middle-aged (mean age < 65 years) [49,50,54–56,59–61] and five were in older adults (mean age > 65 years) [48,53,57,58,62]. Sample sizes ranged from 11 to 150 (total combined data of the 13 studies $n = 336$ [220 women, 116 men]). Participants' age range was 52 to 73 years (mean age 62 ± 6 years) and BMI was 23.5 to 33.1 kg/m^2 (mean BMI 26.85 ± 3.33 kg/m^2). Sex-distribution for included studies was predominately women (71%); 77% of middle-aged and 54% of older adults were women.

Eleven studies evaluated effects of acute AE exercise on BTMs (seven in middle-aged [49,50,55,56,59–61], and four in older adults [48,53,58,62]). Two studies evaluated effects of acute combined RE and impact (middle-aged adults) [49,55], one study evaluated the effects of acute impact exercise alone (middle-aged adults) [54], and one study evaluated the effects of acute RE alone (older adults) [57] on changes in BTMs. Only two studies reported that the exercise was supervised [48,54]. Exercise protocols, blood sampling protocols and effects of acute exercise on BTMs have been described in Table 3 including all reported levels and significant changes.

Nine studies reported that exercise and blood sampling were performed in the morning [49,50,53–56,59,61,62], one was performed in the afternoon [60], and three did not state the time of the day [48,57,58]. Seven studies were performed in the morning following an overnight fast [49,50,53–56,59], one stated at least 12 h of fasting (no indication of time) [57], and five studies were not performed in a fasted state [48,58,60–62]. One study involved a controlled pre-feed [48], and another stated a 2-hour fast after a meal free from milk and cheese [60]. Only three studies reported controlling for exercise on preceding days [54,61,62]. One study mentioned withholding dietary supplements [54]. Post-exercise blood sampling varied greatly from one to four timepoints; four studies taking only immediately post [52,53,55,58,59], the longest taken at 72 h [61,62]. A range of biochemical assays were used to analyse the circulating BTMs including electrochemiluminescence immunoassay (ECLIA), enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and immunoradiometric assay (IRMA) (Table 3).

3.3. Acute aerobic exercise

3.3.1. Effects on BTMs: middle-aged adults

Two studies reported significant increases in ALP immediately following cycling GXTs performed to exhaustion in men and in middle-aged postmenopausal women [59,60]. BALP also increased (range ~ 0.7 to 26%) in women after a cycling GXT to exhaustion, and also after moderate intensity walking (46 min, 3–6 METs) [55,60]. Three studies reported a significant increase in tOC (range ~ 13.4 to 18.8%) in men who cycled (GXT to exhaustion; and 75% VO^2_{Peak}, 30 min), and in middle-aged postmenopausal women who jogged (50% HR_{Max} reserve, 45 min) [49,59,61]. However, three cycling studies reported no change in tOC, one in men (90–95% HR_{Peak}, 30 min) and two in middle-aged postmenopausal women (70–75% VO^2_{Peak}, 30 min; GXT to exertion) [50,56,60]. No significant change was reported in P1NP after cycling in middle-aged postmenopausal women (70–75% VO^2_{Peak}, 30 min) [56] or in men (90–95% HR_{Peak}, 30 min) [50]. Acute AE was also reported to have no effect on PICP in middle-aged postmenopausal women after jogging (50% HR_{Max} reserve, 45 min) [61].

One study reported that acute AE significantly increased (~16.6%) β-CTX after cycling in men (90–95% HR_{Peak}, 30 min), however, there was no change in β-CTX after cycling (75% VO^2_{Peak}, 30 min) or CTX after walking (3–6 METs, 46 min) in middle-aged postmenopausal women [50,55,56]. Two studies measured ICTP with no significant changes in middle-aged postmenopausal women after jogging (50% HR_{Max} reserve, 45 min) or cycling (to exertion, GXT) [60,61]. SCL was reported to increase following brisk walking in middle-aged postmenopausal women (3–6 METs, 46 min) [55].

3.3.2. Effects on BTMs: older adults

ALP significantly increased in men and women immediately following a treadmill GXT (stopped at 75–85% HR_{Max}) [58]. BALP also significantly increased (~12%) immediately following a treadmill GXT (to exertion), but only in men and women who were classed as moderately active (classified using a physical activity questionnaire) and not active based on baseline exercise levels [53]. Two studies reported that tOC did not change in women after walking (50% HR_{Max} reserve, 90 min) or in men and women after a treadmill GXT (to exhaustion) [53,62]. PICP was reported to increase in women after walking (50% HR_{Max} reserve, 90 min) [62].

Wherry et al. [48] reported significant increases (range 34.6 to 77.3%) in CTX levels at all post-exercise time points (peak, 15, 30, 45 and 60 min) in men and women who walked at moderate intensity (70–80% HR_{Max}, 60 min). In contrast, Maimoun et al. [53] reported no significant change in men and women following a maximal GXT (treadmill). Thorsen et al. [62] reported a significant decrease (~13.8%) in 1CTP levels at 1 h, but a significant increase (~15.5%) in levels at 72 h post brisk walking (50% HR_{Max} reserve, 90 min).

3.4. Acute resistance with and without impact, or impact alone exercise

3.4.1. Effects on BTMs: middle-aged and older adults

The effect of acute RE with and without impact exercises, versus impact only exercise on BTMs greatly varied with a limited number of studies measuring the same BTMs. In studies involving RE + impact, no change was reported in BALP in middle-aged postmenopausal women, or in tOC in middle-aged men [49,55]. On the contrary, impact-only exercise (three forms of jumping, *see* Table 3) significantly increased tOC (double jump group) and P1NP (all groups) immediately post, but at 2-h tOC significantly decreased (all groups), with P1NP also reducing (non-significant) to below baseline levels [54]. The drop in tOC (significant) and P1NP (non-significant) to below baseline levels was consistent with the control group in that study [54]. CTX was the only consistent measured bone resorption marker shown to decrease following RE + impact and impact-alone protocols in middle-aged women [54,55]. However, in the impact-alone study, the significant

Table 3
Study characteristics and outcomes.

Study details Author, journal (year)	Study design	Participants Sample (n, M/F) Age (years) Height (cm) Weight (kg) or BMI (kg/m^2) mean ± SD	Acute exercise bout T: type D: duration I: intensity S&R: sets and reps	Blood sampling and assay protocol T: timepoints C: controls B: bone turnover markers measured A: assay protocol	Main findings Effects of acute exercise compared to baseline
Middle-aged adults mean age 50 to 65 years					
Gombos et al. BMC Musculoskeletal Disorders (2016) [54]	RCT	Healthy middle-aged women *n* = 150 RE + IMP (*n = 50*) 60.2 ± 6.9 yrs. 162.6 ± 9.8 cm 69.7 ± 11.8 kg 26.3 ± 5.4 kg/m^2 −2.2 ± 0.7 T-score AER (n = 50) 58.7 ± 6.3 yrs. 159.6 ± 6.4 cm 72.7 ± 14.8 kg 27.2 ± 6.1 kg/m^2 −1.9 ± 0.9 T-score CON (n = 50) 57.8 ± 8.4 yrs. 161.7 ± 5.0 cm 69.5 ± 13.0 kg 28.1 ± 3.9 kg/m^2 −2.1 ± 0.7 T-score (T-score site not stated)	Randomised to: 1. RE + IMP T: resistance exercises of large muscle groups, core stabilisation and impact D: 5 min warm up, 30 min resistance exercises, 8 min cool down I: not stated S&R: 3 sets of 4 to 8 reps 2. AER T: brisk walking (W) at 100 steps/min D: 46 min I: moderate intensity at 3 to 6 METs 3. CON T: nil intervention	T: baseline, post ex (+0 to 5 min) C: OFT, AM B: CTX, BALP and SCL A: CTX - ECLIA, BALP - photometric assay, SCL - ELISA	Post exercise at 0 to 5 min (all mean ± SD) ↑ BALP AER only RE + IMP 41.7 ± 12.8 to 41.8 ± 12.0% AER 41.8 ± 7.6 to 42.1 ± 8.4%* CON 42.2 ± 10.4 to 42.1 ± 10.2% ↓ CTX RE + IMP only RE + IMP 303.6 ± 156.8 to 276.4 ± 143.6 pg/mL** AER 247.3 ± 106.2 to 253.9 ± 107.5 pg/mL CON 259.1 ± 110.2 to 256.7 ± 111.2 pg/mL ↑ SCL AER only RE + IMP 26.8 ± 14.0 to 29.8 ± 15.7 pmol/L AER 23.6 ± 10.0 to 29.9 ± 10.8 pmol/L** CON 24.0 ± 8.8 to 24.2 ± 8.8 pmol/L
Levinger, I., et al. Osteoporos Int (2011) [48]	Randomised parallel design	Middle-aged obese, men *n* = 28 AER (n = 13) 52.8 ± 5.41 yrs. 174.9 ± 6.49 cm 100.5 ± 18.75 kg 32.7 ± 5.41 kg/m^2 RE + IMP (*n = 15*) 52.1 ± 6.97 yrs. 177.7 ± 5.03 cm 99.2 ± 13.94 kg 31.5 ± 4.65 kg/m^2	Randomised to: 1. AER T: cycling D: 45 min I: 75% of VO^2_{Peak} 2. RE + IMP T: resistance exercise including power leg press and jumping D: 45 min I: 70 to 75% of 1RM S&R: 2 × 5 sets of 8 leg press, 3 × 5 sets of 10 jumps	T: baseline, post ex (+0, 30, 60 and + 120 min) C: OFT, AM B: tOC (and ucOC) A: tOC - CLIA, ucOC - ECLIA	Post exercise to peak (all mean ± SD) ↑ tOC AER group only AER 5.32 ± 2.89 to 6.08 ± 3.51 ng/mL ** RE + IMP 4.82 ± 1.63 to 5.01 ± 2.03 ng/mL ↑ ucOC AER group only AER 4.64 ± 3.03 to 5.08 ± 3.5 ng/mL ** RE + IMP 3.93 ± 1.53 to 3.99 ± 1.51 ng/mL
Levinger, I., et al. JBMR (2014) [49] Additional analysis for PINP and β-CTX reported in Levinger, I., et al. BoneKEy Rep (2015) [50]	Non-randomised, case-controlled crossover	Middle-aged obese, non-diabetic men *n* = 11 58.1 ± 7.29 yrs. 176 ± 5.64 cm 102.5 ± 12.93 kg 33.1 ± 4.64 kg/m^2	Completed both 1. CON T: complete rest D: 30 min 2. AER T: cycle ergometer, high intensity exercise D: 30 min I: 4 min warm up @ 50 to 60% HR_{Peak} & cycling as:	T: baseline, post ex (+0, 30 and 60 min) C: OFT, AM B: tOC (and ucOC, ucOC/tOC) and P1NP and β-CTX A: tOC - automated immunoassay, ucOC - automated immunoassay following hydroxyl-apatite slurry, P1NP - ELCIA, β-CTX - ECLIA	Post exercise to peak (all mean ± SEM) NC tOC AER 18.2 ± 1.4 to 18.61 ± 1.48 ng/mL NC P1NP AER 36.1 ± 1.3 to 37.09 ± 1.56 μ/L^{-1}

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Table 3 (*continued*)

Study details Author, journal (year)	Study design	Participants Sample (n, M/F) Age (years) Height (cm) Weight (kg) or BMI (kg/m^2) mean ± SD	Acute exercise bout T: type D: duration I: intensity S&R: sets and reps	Blood sampling and assay protocol T: timepoints C: controls B: bone turnover markers measured A: assay protocol	Main findings Effects of acute exercise compared to baseline
			4 × 4 min @ 90 to 95% HR_{Peak} 2 min active recovery @ 50 to 60% HR_{Peak}		↑ β-CTX (~16%) AER 306.5 ± 41 to 357.45 ± 50.33 μ/L^{-1}** ↑ ucOC (~2.1%) AER 10.6 ± 0.8 to 11.21 ± 0.69 ng/mL* ↑ ucOC/OC (~1.9%) AER 58.9 ± 2.0 to 62.1 ± 1.9%*
Levinger, I., et al. Physiol Rep (2016) [55]	Randomised, case-controlled crossover	Postmenopausal women n = 10 62.8 ± 8.22 yrs. 161.2 ± 5.06 cm 73.6 ± 10.75 kg 28.3 ± 4.11 kg/m^2	Completed both: 1. CON T: complete rest D: 30 min *2.* AER T: cycle ergometer D: 30 min I: 70 to 75% of $VO^2{}_{Peak}$	T: baseline, post ex (+0, 30, 60 and 120 min) C: OFT, AM B: β-CTX, P1NP, tOC (and ucOC) A: β-CTX - ECLIA, P1NP - ELCIA, tOC - automated immunoassay, ucOC - automated immunoassay following hydroxyl-apatite slurry	Post exercise to peak (all mean ± SD) NC tOC 28.1 ± 8.6 to 28.38 ± 8.76 ng/mL NC P1NP 67.2 ± 7.6 to 62.6 ± 19.09 μ/L^{-1} NC β-CTX 429.3 ± 40.1 to 470.1 ± 145.61 μ/L^{-1} ↑ ucOC 13.83 ± 6.71 to 15.04 ± 7.35 ng/mL**
Prawiradilaga et al. Biol Sport (2020) [53]	RCT-crossover	Healthy, sedentary postmenopausal women *n* = 29 60.0 ± 5.6 yrs. 165.2 ± 5.4 cm 65.8 ± 7.7 kg 24.1 ± 2.5 kg/m^2	Each participant performed in a random order 3 high-impact exercise trials and CON Session 1- IMPACT T: 7 min low impact warm up on a gymnastic mat, then counter movement jump (CMJ) vertical jump with two leg launch and land. Session 2- IMPACT T: 7 min low impact warm up on a gymnastic mat then drop jump (DJ) from a 32 cm box, the landing continued into a vertical two-leg jump Session 3- IMPACT T: 7 min low impact warm up on a gymnastic mat then DDJ (above) but performed diagonally forward 45° For session 1 to 3: D: not stated I: not stated S&R: 6 sets of 10 reps interspersed with 90 s rest	T: baseline, post ex (immediately after and + 2 h) C: AM, OFT, nil vigorous exercise preceding 48 h, dietary supplements withheld B: P1NP, tOC, CTX A: P1NP - CLIA, tOC - CLIA, CTX - CLIA	Post exercise at 0 min (all mean ± SE) P1NP ↑ for CMJ, DJ and DDJ, NC for CON CMJ 70.2 ± 5.6 to 75.6 ± 6.3 μg/L** DJ 71.0 ± 5.5 to 77.6 ± 5.8 μg/L** DDJ 73.0 ± 6.3 to 80.8 ± 6.8 μg/L** CON 71.9 ± 5.3 to 70.1 ± 5.6 μg/L tOC ↑ for DJ only NC for CMJ, DDJ and CON CMJ 31.2 ± 2.3 to 32.2 ± 2.4 μg/L DJ 30.7 ± 2.2 to 32.4 ± 2.5 μg/L* DDJ 30.6 ± 2.2 to 31.8 ± 2.3 μg/L CON 31.1 ± 2.1 to 30.0 ±

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Table 3 (*continued*)

Study details Author, journal (year)	Study design	Participants Sample (n, M/F) Age (years) Height (cm) Weight (kg) or BMI (kg/m^2) mean ± SD	Acute exercise bout T: type D: duration I: intensity S&R: sets and reps	Blood sampling and assay protocol T: timepoints C: controls B: bone turnover markers measured A: assay protocol	Main findings Effects of acute exercise compared to baseline
			Session 4 CON T: complete rest		2.0 μg/L NC for CTX all sessions CMJ 636.0 ± 83.4 to 635.5 ± 80.3 ng/L DJ 645.2 ± 88.3 to 666.2 ± 91.0 ng/L DDJ 612.8 ± 85.9 to 632.8 ± 85.4 ng/L CON 590 ± 73.6 to 582.4 ± 74.4 ng/L Post exercise at 2 h NC for P1NP all sessions CMJ 70.2 ± 5.6 to 68.7 ± 6.0 μg/L DJ 71.0 ± 5.5 to 67.5 ± 6.0 μg/L DDJ 73.0 ± 6.3 to 70.2 ± 6.0 μg/L CON 71.9 ± 5.3 to 70.6 ± 5.4 μg/L tOC ↓ for CMJ, DJ and CON only CMJ 31.2 ± 2.3 to 28.9 ± 2.2 μg/L** DJ 30.7 ± 2.2 to 28.3 ± 2.5 μg/L** DDJ 30.6 ± 2.2 to 29.2 ± 2.2 μg/L CON 31.1 ± 2.1 to 28.1 ± 2.0 μg/L** CTX ↓ for all sessions CMJ 636.0 ± 83.4 to 527.9 ± 65.7 ng/L** DJ 645.2 ± 88.3 to 525.5 ± 69.0 ng/L** DDJ 612.8 ± 85.9 to 519.0 ± 69.1 ng/L** CON 590 ± 73.6 to 501.7 ± 65.8 ng/L**
Rudberg et al. Calcif Tissue Int (2000) [59]	Non-randomised, single arm	Postmenopausal women n = 8 57 ± 4 yrs. 164 ± 5 cm 69.5 ± 9.6 kg 25.9 ± 3.6 kg/m^2	1. AER T: cycle GXT D: average duration of test was 24 min I: workload ↑ by 30 W every 6 min (start 30 W) until exhaustion	T: baseline, post ex (+0 and 20 min) C: PM, 2 h post feed (NFT) B: ICTP, ALP total, B-ALP 1 and 2 and tOC A: ICTP - RIA, B-ALP 1 and 2 - HPLC, tOC - RIA, ALP total - unclear	Post exercise at 0 min (all mean ± SD) ↑ ALP total, ALP B/I, AP B1, ALP B2, ALP L1, ALP L3 NC all other markers ALP total 3.08 ± 0.73 to 3.40 ± 0.70 ukat/L**

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Table 3 (*continued*)

Study details Author, journal (year)	Study design	Participants Sample (n, M/F) Age (years) Height (cm) Weight (kg) or BMI (kg/m^2) mean ± SD	Acute exercise bout T: type D: duration I: intensity S&R: sets and reps	Blood sampling and assay protocol T: timepoints C: controls B: bone turnover markers measured A: assay protocol	Main findings Effects of acute exercise compared to baseline
					ALP B/I 0.12 ± 0.06 to 0.15 ± 0.07 ukat/L** ALP B1 0.50 ± 0.18 to 0.63 ± 0.21 ukat/L** ALP B2 1.18 ± 0.45 to 1.49 ± 0.45 ukat/L** ALP B1/B2 0.43 ± 0.07 to 0.43 ± 0.08% tOC 3.2 ± 1.4 to 2.9 ± 1.0 μg/L ICTP 2.8 ± 0.9 to 2.7 ± 0.8 μg/L Post exercise at 20 min NS all markers ALP total 3.08 ± 0.73 to 3.32 ± 0.84 ukat/L ALP B/I 0.12 ± 0.06 to 0.14 ± 0.07 ukat/L ALP B1 0.50 ± 0.18 to 0.61 ± 0.20 ukat/L ALP B2 1.18 ± 0.45 to 1.46 ± 0.49 ukat/L ALP B1/B2 0.43 ± 0.07 to 0.43 ± 0.09% tOC 3.2 ± 1.4 to 3.5 ± 1.2 μg/L ICTP 2.8 ± 0.9 to 2.4 ± 0.5 μg/L
Thorsen et al. Eur J Exp Musculoskel Res (1995) [60]	Single arm	Early postmenopausal women $n = 15$ 55 ± 3.87 yrs. 165 ± 3.87 cm 65.0 ± 7.75 kg 23.7 ± 2.32 kg/m^2 1.06 ± 0.03 g/cm^2 Total BMD	1. AER T: jogging (6 degrees) D: 45 min I: 50% of $VO^2{}_{Max}$ estimated by 50% of HR_{Max} reserve	T: baseline, post ex (+1, 24 and 72 h) C: no-exercise for 3 days prior/post, AM, NFT B: P1CP, ICTP, and tOC A: P1CP - RIA, ICTP - RIA, tOC - IRMA	Post exercise at 1 h (all mean ± SEM) ↑ tOC tOC 4.8 ± 0.4 to 5.7 ± 0.5 μg/L** PICP 129 ± 15 to 128 ± 15 μg/L ICTP 2.33 ± 0.25 to 2.48 ± 0.17 μg/L Post exercise at 24 h NC tOC, PICP or ICTP tOC 4.8 ± 0.4 to 5.5 ± 0.6 μg/L PICP 129 ± 15 to 134 ± 13 μg/L ICTP 2.33 ± 0.25 to 2.61 ± 0.21 μg/L Post exercise at 72 h NC tOC, PICP or ICTP

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Table 3 (*continued*)

Study details Author, journal (year)	Study design	Participants Sample (n, M/F) Age (years) Height (cm) Weight (kg) or BMI (kg/m^2) mean ± SD	Acute exercise bout T: type D: duration I: intensity S&R: sets and reps	Blood sampling and assay protocol T: timepoints C: controls B: bone turnover markers measured A: assay protocol	Main findings Effects of acute exercise compared to baseline
					tOC 4.8 ± 0.4 to 5.3 ± 0.5 μg/L PICP 129 ± 15 to 140 ± 12 μg/L ICTP 2.33 ± 0.25 to 2.61 ± 0.21 μg/L
Zerath et al. Med Sci Sp Exerc. (1997) [58]	Single arm	Healthy active males *n* = 24 62.3 ± 5.39 yrs. 172.3 ± 5.39 cm 71.9 ± 8.33 kg	1. AER T: maximal cycle GXT D: ~10 min I: increased by 20 W every 2 min until exhaustion	T: baseline, post ex (+0 to 1 min) C: AM, OFT B: ALP, tOC A: ALP - autoanalyzer, tOC - RIA	Post exercise at 0–1 min (all mean ± SEM) ↑ ALP and tOC ALP 41.7 ± 3.5 to 47.8 ± 3.7 μL/L^{-1}* tOC 6.18 ± 0.44 to 7.01 ± 0.36 ng·mL^{-1}*
Older adults mean age >65 years					
Aly et al. Geriatric Med and Care (2017) [57]	Single arm	# Elderly men & women *n* = 40 (26/14) 66.2 ± 6.3 yrs. 163.64 ± 26.44 cm 71 ± 5.5 kg 25.24 ± 2.15 kg/m^2	1. AER T: treadmill GXT D: ~10 min I: 3 min warm up @ 40% age predicted HR_{Max}, gradual increase of exercise intensity until reaching 75 to 85% calculated HR_{Max}	T: baseline, post ex (+10 to 30 s) C: NFT B: ALP A: ALP - kinetic assay	Post exercise at 10–30 s (all mean ± SD) ↑ ALP ALP 63.76 ± 19.24 to 75.4 ± 21.9 **
Kim et al J Exerc Nutr Biochem (2014) [56]	Single arm	Elderly osteopenic women *n* = 11 (5 osteoporotic) 68.18 ± 3.19 yrs. 151.24 ± 2.94 cm 54.29 ± 5.21 kg 23.73 ± 2.07 kg/m^2 −2.51 ± 0.47 T-score *T-score is whole body	All participants completed in the same order (1 week apart) 1. CON T: nil intervention, rest in chair D: not stated 2. RE T: pilates exercises D: ~70 min including warm up and 50 min of pilates exercises I: warm up (RPE 9 to 12), pilates exercises (RPE 10 to 14) S&R: not stated	T: baseline, post ex (+0 and 60 min) C: At least 12 h of fasting B: ALP A: ALP - modular DDP analysis	Post exercise at 0 min (all mean ± SD) CON 60.2 ± 13.3 to 60.2 14.0 RE 59.**1** ± 14.0 to 58.5 ± 14.2 Compared to baseline at 60 min ↓ ALP at 60 min CON 60.2 ± 13.3 to 58.9 ± 13.6 RE 59.1 ± 14.0 to 57.1 ± 13.8**
Maimoun et al. Br J Sp Med (2005) [52] *Follow up study*: Maimoun et al. J Sci & Sp. Med (2009) [51]	Non-randomised, single arm, group comparison	Active elderly *n* = 21 (11/10) 73.3 ± 9.1 yrs. 166.3 ± 9.2 cm 65.8 ± 13.2 kg 23.6 ± 2.9 kg/m^2 Follow up study *n* = 45 Active *n* = 18 (10/8) 71.7 ± 8.6 yrs. 166.9 ± 9.3 cm 66.1 ± 13.3 kg 23.5 ± 2.9 kg/m^2 Moderately active	1. AER T: maximal treadmill GXT at preferred walking speed including a warm up walking at 0% grade, followed by 1 to 2% gradient increase until exhaustion D: 5 min warm up followed by maximal incremental test of 8 to 12 min duration I: maximal	T: baseline, post ex (+0 min) C: OFT, AM B: CTX, tOC and BALP A: CTX - ELISA, tOC - IRMA, BALP - IRMA	2005 study post exercise at 0 min (all mean ± SD) NC all markers CTX 5998 ± 3045 to 5959 ± 2866 pmol/L^{-1} tOC 12.7 ± 5.5 to 12.5 ± 5.3 ng/mL^{-1} BALP 13.1 ± 4.8 to 13.2 ± 4.7 ng/mL^{-1} 2009 study post exercise at 0 min ↑ BALP for moderately active group only, NC all other markers

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Study details Author, journal (year)	Study design	Participants Sample (n, M/F) Age (years) Height (cm) Weight (kg) or BMI (kg/m^2) mean ± SD	Acute exercise bout T: type D: duration I: intensity S&R: sets and reps	Blood sampling and assay protocol T: timepoints C: controls B: bone turnover markers measured A: assay protocol	Main findings Effects of acute exercise compared to baseline
		n = 18 (10/8) 71.9 ± 7.3 yrs. 166.6 ± 7.8 cm 70.7 ± 12.7 kg 25.3 ± 3.2 kg/m^2			Active CTX 5998 ± 3045 to 5959 ± 2866 pmol/L^{-1} tOC 12.7 ± 5.5 to 12.5 ± 5.3 ng/mL^{-1} BALP 13.1 ± 4.8 to 13.2 ± 4.7 ng mL^{-1} Moderately active CTX 5595 ± 2460 to 5385 ± 2201 pmol L^{-1} tOC 12.2 ± 4.5 to 12.6 ± 3.9 ng mL^{-1} BALP 11.6 ± 2.9 to 13.0 ± 4.1 ng mL^{-1}*
Thorsen et al. Calcific Tissue Int (1996) [61]	Single arm	Postmenopausal women *n* = 12 68 ± 3.46 yrs. 167 ± 3.46 cm 71.2 ± 7.97 kg 25.3 ± 2.08 kg/m^2 1.05 ± 0.03 g/cm^2 total BMD	1. AER T: brisk walking (−2 degrees) D: 90 min I: 50% of VO^2_{Max} estimated by 50% of HR_{Max} reserve	T: baseline, post ex (+1, 24 and 72 h) C: AM, NFT, no-exercise for 3 days prior or post B: ICTP, tOC, P1CP A: ICTP - RIA, tOC - IRMA, P1CP - RIA	Post exercise at 1 h (all mean ± SEM) NC tOC or PICP, ↓ in ICTP tOC 7.3 ± 0.5 to 7.4 ± 0.4 μg/L PICP 139 ± 11 to 132 ± 10 μg/L ICTP 2.88 ± 0.12 to 2.48 ± 0.19 μg/L* Post exercise at 24 h NC tOC or ICTP, ↑ PICP tOC 7.3 ± 0.5 to 6.9 ± 0.5 μg/L PICP 139 ± 11 to 155 ± 13 μg/L** ICTP 2.88 ± 0.12 to 3.18 ± 0.32 μg/L Post exercise at 72 h NC tOC, ↑ PICP, ↑ in ICTP tOC 7.3 ± 0.5 to 7.4 ± 0.8 μg/L PICP 139 ± 11 to 157 ± 11 μg/L** ICTP 2.88 ± 0.12 to 3.33 ± 0.21 μg/L**
Wherry et al. Med & Sci Sports Ex. (2019) [47]	Non-randomised, uncontrolled crossover	Healthy recreationally active older adults n = 12 (5/7) 67 ± 5 yrs. 1.7 ± 0.1 m 67.7 ± 15.9 kg −1.6 ± 0.6 T-score (T-score is femoral neck)	Two acute bouts of treadmill walking performed 1 to 4 weeks apart under cool and warm conditions 1. AER T: treadmill walking D: 60 min (+ 5 min warm up and 5 min cool down) I: 70 to 80% of HR_{Max}	T: baseline, post ex (peak, +15, 30, 45 and 60 min) C: NFT, controlled pre-feed B: CTX A: CTX - chemiluminescence	Post exercise at peak (all mean ± SD) ↑ CTX both conditions Cool 0.255 ± 0.14 to 0.355 ± 0.17 ng/mL* Warm 0.255 ± 0.14 to 0.309 ± 0.114 ng/mL*

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Table 3 (*continued*)

Study details Author, journal (year)	Study design	Participants Sample (n, M/F) Age (years) Height (cm) Weight (kg) or BMI (kg/m^2) mean ± SD	Acute exercise bout T: type D: duration I: intensity S&R: sets and reps	Blood sampling and assay protocol T: timepoints C: controls B: bone turnover markers measured A: assay protocol	Main findings Effects of acute exercise compared to baseline
					Post exercise at 15 min ↑ CTX both conditions Cool 0.255 ± 0.14 to 0.353 ± 0.163 ng/mL* Warm 0.255 ± 0.14 to 0.353 ± 0.163 ng/mL* Post exercise at 30 min ↑ CTX both conditions Cool 0.255 ± 0.14 to 0.375 ± 0.16 ng/mL* Warm 0.255 ± 0.14 to 0.348 ± 0.115 ng/mL* Post exercise at 45 min ↑ CTX both conditions Cool 0.255 ± 0.14 to 0.364 ± 0.184 ng/mL* Warm 0.255 ± 0.14 to 0.365 ± 0.127 ng/mL* Post exercise at 60 min ↑ CTX both conditions Cool 0.255 ± 0.14 to 0.400 ± 0.177 ng/mL* Warm 0.255 ± 0.14 to 0.391 ± 0.129 ng/mL* *changes not different between conditions

Keywords: RCT, randomised controlled trial; M, male; F, female; PoM, post-menopause; FT, fasting; OFT, overnight fasted; NFT, not fasted; AM, performed in morning; PM, performed in afternoon; RE, resistance exercise; RE+IMP, resistance and impact exercise; IMPACT, impact only exercise; 1RM, one repetition maximum; AER, aerobic exercise, CON, control; GXT, graded exercise test; ALP, alkaline phosphatase; BALP, bone specific alkaline phosphatase; PICP, Procollagen I Carboxyterminal Propeptide; P1NP, Procollagen Type 1 N Propeptide; OC, osteocalcin; ucOC, undercarboxylated osteocalcin; CTX, C-Terminal Crosslinked Telopeptide of Type I Collagen; ICTP, Cross-Linked Carboxyterminal Telopeptide of Type I Collagen; SCL, sclerostin; ECLIA, electrochemiluminescence immunoassay; ELISA, enzyme-linked immunosorbent assay; CLIA, chemiluminescent immunometric assay; RIA, radioimmunoassay; HPLC, high-performance liquid chromatography; IRMA, immunoradiometric assay.

NC, no change compared to baseline or control; ↑, significant increase compared to baseline or control; ↓, significant decrease compared to baseline or control, $^*p \leq 0.05$, $^{**}p \leq 0.01$.

decrease at 2-h post (not immediately after) was not significantly different to the control group [54]. Only one study investigated acute RE in older women [57] and reported a significant decrease in ALP; no other BTMs were measured in this study.

4. Discussion

We report that a) BTM responses to acute exercise vary between middle- and older-aged adults and that the BTM responses may be b) sex-specific and c) altered by exercise mode, intensity and duration. Additionally, responses to acute exercise stimuli may be d) BTM-specific, with some markers being more sensitive than others to the same stimuli. We identified a major gap in the current field with a small number of studies investigating acute effects of exercise on BTMs in middle-aged adults ($n = 8$), and even an fewer number in older-adults ($n = 5$).

The application of mechanical stress (i.e. exercise) to the skeleton can preserve and increase BMD, serving as a key intervention in the prevention and management of osteoporosis [8–10]. The effect of chronic, long-term exercise training on BMD in older adults is well established, shown to be modality- and intensity-dependent [9,40,63,64]. Evidence suggests that walking is of limited value for improving bone health if prescribed without additional load bearing exercises [37,40,63,65–67]. It is well accepted that RE with weight bearing and high impact is safe and effective to optimise bone health in older adults, as they result in high strain rates and peak forces and, reduce falls and fractures [7,9,36,38,68]. In fact, high-velocity power and rapid concentric contractions (inducing higher strain rates on bone) is beneficial for functional performance (i.e. chair rise) in older adults [69–71]. Additionally, regular weight-bearing impact, applied in multidirectional patterns, promotes bone maintenance/preservation [63,72]. While the evidence is clear from chronic, long term, exercise training studies what characteristics exercise protocols should consist of for beneficial effects on bone health in adults, the effects of acute exercise are unclear. Available data are conflicting and, as it is not appropriate to measure BMD after a single session, BTMs are used as a surrogate measure [42]. Whether various modes of acute exercise with different modifiable characteristics alter bone metabolism differently in middle and older adults is underexplored.

4.1. Age and sex-specific effects on BTM responses to acute exercise

Based on this review, while acute exercise is sufficient to detect responses in BTMs, these responses may be age- and sex-specific, highlighting some possible consideration in the design of future acute exercise studies. For instance, all AE exercise studies investigating the tOC and BALP response in older adults (men and women) report no change after exercise, but some studies in middle-aged adults (men and women) report increases [49,53,55,59–61]. Conversely, ALP appears to have similar sensitivity in middle and older aged men and women [50,58–60] and resorption markers CTX (men and women) and ICTP (women only) appear to increase in older adults, but not middle-aged [48,55,60–62]. Lastly, tOC and β-CTX responses to AE also appears to be more sensitive in middle-aged men than women, suggesting a possible sex-specific response [49,56,59–61]. Differences in BTM responses between middle- and older-aged adults could be multifactorial, explained by age-related alterations to bone composition and hence bone turnover, and in women, menopausal effects, possibly altering the bone response [6,73–77]. Indeed, underlying bone pathophysiology is different in middle-aged vs older women who, are known to have elevated bone turnover rates, possibly explaining differences in responses [6,78]. Given bone resorption was not significantly altered in some of these studies in women [55,56,60,61] may in fact, be beneficial (not stimulating further the negative balance of the remodelling process), however this is poorly understood and warrants further exploration.

Of note, at baseline, some studies did not report/screen for BMD and/or T-score, as adults are known to be affected by age-related bone composition alterations, particularly women, this should be considered. Some studies excluded individuals with osteoporosis [49,50,53,54], whereas others included adults with osteopenia/osteoporosis [48,55,57], possibly influencing BTM responses [79]. Some studies in older adults pooled men and women data together [48,58], only one confirming no sex-interaction in BTM responses [53]. As older women are known to have different rates of bone turnover and consequently accelerated bone loss compared to men, bone responses may be altered (or attenuated) thus, men and women should be handled separately, or sensitivity tests performed [35,73–77,79].

4.2. BTM responses modulated by exercise mode, intensity, and duration

This review summarises that BTM responses to acute exercise may be modulated by the specific characteristics of the exercise protocol used. For instance, a majority of studies report no change in tOC following AE regardless of intensity (low, moderate, high) [50,53,56,60,62]. However, tOC may be more sensitive only to AE that incorporates loads of greater ground-reaction force increasing in one study after jogging, but not after the majority of studies including cycling or walking protocols [50,53,56,60–62]. Whereas, ALP, BALP and PICP increase after cycling and walking, suggesting these markers have higher sensitivity to AE with lower impact [53,55,58–60,62]. Indeed, in three separate studies in middle-aged men utilising cycling protocols the tOC response was different, increasing only after moderate intensity cycling (30 min) and a short duration maximal exertion GXT, but not after high-intensity interval exercise (30 min) [49,50,59]. This suggests that exercise intensity and duration may be important, but there may be other possible modulating effects on the tOC response, which should be further explored. Markers reflecting bone resorption, CTX and ICTP appear to be more sensitive to AE protocols that are longer (≥60 min), not shorter duration (<45 min) [48,53,55,60–62]. Whereas, β-CTX (a different fragment of CTX) responds differently to cycling exercise of same duration (30 min), increasing only after high-intensity, but not moderate-intensity cycling, suggesting that in this instance, intensity may be important [50,56].

Despite the mounting evidence for the use of RE combined with weightbearing and impact loads distributed in dynamic and novel patterns for optimising bone health effects, little is known about the acute effects and available studies investigating these characteristics is limited. Based on this review, RE with impact does not stimulate a response in markers reflecting bone formation [49,55]. However, one study measured BALP only immediately post exercise [55], the other measured tOC only up to 2-h, possibly missing the kinetic response [49]. Direct comparison of these study protocols is difficult, one study used core stabilisation bodyweight exercises with small impact exercises (steps, hopping) [55], the other study used power leg press RE (70 to 75% maximal strength) with high impact jumping, thus the impact and mechanical strain load on bone would be very different [49]. However, it does appear that high impact exercise alone and RE alone is sufficient to detect a response in BTMs of formation. Indeed, ALP was decreased in one study following a RE regimen of pilates exercises, however, whether this is truly indicative of a bone-response is unclear, and other BTMs were not measured [42,57,80]. Of note, only the study investigating impact alone using three sessions each containing a different form of jumping, reported increases of tOC and P1NP. P1NP increased for all jumping protocols, but tOC was only increased in the session where participants dropped from a height to an explosive vertical jump, not from jumping directly from the floor [54]. Highlighting that, P1NP may be more sensitive than tOC to impact exercise, and that the tOC-specific response may require greater impact loads (ground reaction force) combined with high explosive movements to elicit a response. Based on these studies it appears that CTX decreases with RE combined with impact, and with impact alone protocols [54,55]. However, while both

of these studies were RCTs, the impact only study which was crossover in design report that CTX decreases also in the control condition [54]. This decrease was not different to the decrease seen post the impact exercise, indicating that CTX is affected by circadian/diurnal effects [54,81].

Altogether, the evidence from this review, and from the literature demonstrates that exercise intensity, dynamic, and novelty of new loads (non-habitual nature) placed on the skeleton are important characteristics influencing the bone-exercise response [16,23,24,82,83]. However, only three studies included participants' baseline fitness in the selection criteria [48,49,54]. Three state [60–62] participants were non-regular exercisers, but one reports participants regularly cycling (1-6 km/day, few days a week) [60]. As habitual exercise was not considered in a majority of studies, protocols may lack in specificity, and although some used prior testing to define exercise intensity their protocols possibly lack in novelty of new load [12,24,84]. Indeed, one interesting concept, explored by one study, was the possible effect on the BTM response based on the participants baseline fitness, whereby BALP was only shown to be significantly increased with AE exercise when older adults were further stratified into moderately active, or active groups [53]. This possibly suggests that the BALP response in older adults may be dampened, modulated by the participants' baseline fitness, supporting the principle that bone cells have a threshold level of adaptation and the need for consideration of individualised, progressive (graded, based on baseline fitness) and novelty in protocol loads, discussed earlier [12,24,84]. This should be further explored in future research, as it likely impacts/dampens the BTM-response and therefore a skewness in results.

4.3. BTM-specific responses to acute exercise

To understand if different BTMs thought to reflect the same bone turnover phase have different sensitivities to acute exercise we compared study effects where >1 BTM reflecting the same bone formation or resorption phase was measured within the same study. AE appears to have a limited effect on tOC and P1NP, whereas other markers reflecting bone formation namely ALP, BALP and PICP appear to be more sensitive. Altogether, suggesting that tOC may be the least sensitive BTM of formation and supports the notion that these BTMs may represent different phases of osteoblastic function or formation [42]. Indeed, ALP activity includes serum derived from liver and bone, therefore changes in response of ALP may be non-specific to bone, as such BALP is recommended for its increased specificity [42,80].

While AE appears to have a limited effect on tOC, one concept to raise about tOC is that it exists in the circulation in a carboxylated (cOC) form reflecting more bone mineralisation, and undercarboxylated (ucOC) form, considered the more "bio-active" counterpart, acting as a hormone involved in energy metabolism and possibly a role in muscle maintenance and strength [85–91]. When studies measured effects on tOC only, whether there is a shift in favor of cOC, or ucOC, is unclear, as only few studies measured this [49,50,56]. In these studies, ucOC increased even with null change in tOC in two of them [50,56]. Therefore, regarding tOC, there is much more to be understood.

One study measured >1 BTM reflecting resorption, interestingly SCL, a possible promoter of bone resorption, increased following walking, but not CTX [55,92]. Suggesting, SCL may be more sensitive than CTX, however, blood sampling was performed only once (immediately post) possibly missing peak change in CTX. Of note, SCL increases with age and high levels are associated with long-term physical in-activity/immobilisation [93–96]. Additionally, mechanical unloading increases the expression (gene and protein) of SCL, whereas SCL expression decreases with mechanical loading (in-vivo and in-vitro) [97,98]. Therefore, SCL may be an interesting marker to be included in future studies.

The BTM responses following exercise may be too fast to be a result of new protein being synthesized and secreted by bone. However, there are at least two possible explanations for the rapid alteration of circulating BTMs: 1) it is known that bone responds to fluid shifts [99], which occurs during exercise and as such, it is possible that proteins that were already produced are now released into the circulation at a faster rate and 2) it is plausible that the BTMs are stored in other organs, such as the liver [100], and these are released during exercise. These hypotheses should be tested in future studies.

BTMs are highly dynamic and sensitive, however, investigators should consider factors known to influence BTMs in preparation for testing i.e. circadian/diurnal rhythm, feeding, sleep, smoking, menopause age and exercise [42,43,75–77]. Some studies were not performed in the fasted state and/or in the morning [48,58,60–62]. In addition, blood sampling protocols largely differed between included studies, some sampling only immediately post-exercise, others taking multiple samples up to 2-h post-exercise, and others up to 72-h post-exercise. As blood sampling represents only a small "window in time" it is possible that some studies, particularly those that only sampled immediately post-exercise may have missed the peak response of the BTM-kinetics. As such, it is not clear whether there is an "optimal" time to assess BTMs following exercise. It is highly recommended that blood sampling is taken at several time points post-exercise, perhaps immediately after exercise and then every 30–60 min up to 2–3 h post-exercise, to identify the "peak response" of each individual. The data for each time point, in addition to the "peak response" and perhaps the area under the curve should be presented. While there are some ethical considerations for invasive techniques and frequency of venepuncture and/or sampling volume, a better understanding of the time-course response of BTM-kinetics is required. Despite advances in quality assurance, laboratory errors commonly occur in pre-analytical phases i.e. timing of sampling, selection of specimen, collection procedure and, sample transport, temperature and time to storage, thus extra rigor should be employed to ensure accurate and reproducible results [43,44,101,102].

4.4. Limitations and strengths

To our knowledge this is the first systematic review to examine effects of acute exercise on BTMs in adults >50 years of age, highlighting major gaps in the field and considerations for increased rigor in future trials. The current review emphasises that research into the effects of acute exercise on BTMs in middle-aged adults is limited and is even scarcer in older adults. While the number of included studies is low ($n = 13$), it covers the only available research in this area. Several factors limit the generalizability of the findings; a lack of RCTs, low quality of the evidence, small sample sizes, potential bias in the cohorts, large variance in the exercise and blood sampling protocols, and the use of different assays to detect BTMs. The latter is an important factor that may lead to differences in findings between studies as the sensitivity of each assay may vary. In addition, it will be important for future studies to explore the chronic adaptation of BTMs to exercise training, to identify the optimal frequency, intensity and mode of exercise that should be taken to elicit optimal bone responses.

5. Conclusions

Acute exercise is an effective tool to induce changes in serum BTMs, however, the response appears to be exercise modality-, intensity-, age- and sex-specific. Large variability in study populations, exercise and blood sampling protocols explains conflicting results and as such, future studies should include tight control over factors that influence BTMs. Longer sampling periods of BTMs may assist in understanding the BTMs-kinetic responses. Further high-quality acute exercise studies are needed to identify new mechanistic target pathways for therapeutics and optimising exercise prescription for adults.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2020.115766.

Funding

This work was partly funded by the Tom Penrose Community Service Grant from Exercise and Sports Science Australia (ESSA) and the Victoria University 2020 Place-Based Planetary Health Research Grant Program.

Availability of data and material

All data reported in this systematic review are included in this published article.

Code availability

Not applicable.

Author contributions

All authors have made substantial contributions to various elements of the study. CS, AT and JM conducted the literature search, CS and AT examined and performed data extraction of articles. CS designed the figures and tables. All authors contributed to the study design and interpretation of study results. All authors contributed to the writing and reviewing of the manuscript.

Declaration of competing interest

None declared.

Acknowledgements

None to declare.

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Appendix 5 Published version of manuscript: Uncovering the bone-muscle interaction and its implications for the health and function of older adults, the Wellderly Project: a protocol for a randomised, controlled crossover trial

Protocol

Uncovering the Bone-Muscle Interaction and Its Implications for the Health and Function of Older Adults (the Wellderly Project): Protocol for a Randomized Controlled Crossover Trial

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Abstract

Background: Bone and muscle are closely linked anatomically, biochemically, and metabolically. Acute exercise affects both bone and muscle, implying a crosstalk between the two systems. However, how these two systems communicate is still largely unknown. We will explore the role of undercarboxylated osteocalcin (ucOC) in this crosstalk. ucOC is involved in glucose metabolism and has a potential role in muscle maintenance and metabolism.

Objective: The proposed trial will determine if circulating ucOC levels in older adults at baseline and following acute exercise are associated with parameters of muscle function and if the ucOC response to exercise varies between older adults with low muscle quality and those with normal or high muscle quality.

Methods: A total of 54 men and women aged 60 years or older with no history of diabetes and warfarin and vitamin K use will be recruited. Screening tests will be performed, including those for functional, anthropometric, and clinical presentation. On the basis of muscle quality, a combined equation of lean mass (leg appendicular skeletal muscle mass in kg) and strength (leg press; one-repetition maximum), participants will be stratified into a high or low muscle function group and randomized into the controlled crossover acute intervention. Three visits will be performed approximately 7 days apart, and acute aerobic exercise, acute resistance exercise, and a control session (rest) will be completed in any order. Our primary outcome for this study is the effect of acute exercise on ucOC in older adults with low muscle function and those with high muscle function.

Results: The trial is active and ongoing. Recruitment began in February 2018, and 38 participants have completed the study as of May 26, 2019.

Conclusions: This study will provide novel insights into bone and muscle crosstalk in older adults, potentially identifying new clinical biomarkers and mechanistic targets for drug treatments for sarcopenia and other related musculoskeletal conditions.

Trial Registration: Australia New Zealand Clinical Trials Registry ACTRN12618001756213; https://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?id=375925.

International Registered Report Identifier (IRRID): DERR1-10.2196/18777

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(JMIR Res Protoc 2021;10(4):e18777) doi: 10.2196/18777

KEYWORDS

acute exercise; clinical trial; bone; adult; aging; osteocalcin; muscles; sarcopenia; progenitor cells; stem cells

Introduction

Background

Adults reach their peak muscle and bone mass in the third decade of life, after which an age-related loss of skeletal muscle and bone mass occurs [1,2]. Under certain conditions, for reasons that are not fully understood, this loss of bone mass (osteoporosis) and muscle (sarcopenia) is accelerated and, in some cases, occurs concurrently [3-5]. Emerging evidence suggests that this parallel and exponential loss of bone and muscle mass and strength is driven, at least in part, by bone and muscle crosstalk. The skeleton and skeletal muscle are closely linked anatomically, biochemically, and metabolically and modulate each other in endocrine and paracrine manners [6]. Many factors may be involved in this crosstalk, including genetics, changes in vitamin D and parathyroid hormone (PTH) levels, aging, increased levels of systemic and local inflammatory markers (ie, interleukin-6 [IL-6] and tumor necrosis factor), obesity and adipokines, mechanical loading, and altered hormones (ie, osteocalcin, resistin, and myostatin) [7,8]. The exact mechanisms involved in this crosstalk remain partially explored, although it has been proposed that undercarboxylated osteocalcin (ucOC) and possibly circulating osteoprogenitor (COP) cells may be mediators [6,9-12].

Serum total osteocalcin (tOC) is an osteoblast-specific secreted protein within the circulation that can be present in two major forms, as follows: γ-carboxylated osteocalcin (cOC) and ucOC lacking γ-carboxylation at one or more sites [13]. cOC, which is predominantly located in bone, is at least partly involved in bone mineralization, whereas ucOC has been shown to be involved in glucose metabolism—at least in mice—with new evidence suggesting a role in influencing muscle mass and strength [10,14-24]. Osteocalcin-deficient mice have reduced muscle mass and strength [17], and lower ucOC levels following hindlimb immobilization in rats are associated with reduced muscle mass and muscle force [25]. Treatment with ucOC can increase the cross-sectional area of the extensor digitorum longus, improve grip strength in mice, and stimulate myotube formation in C2C12 myoblast cultures in vitro [16]. In humans, we and others have shown that exercise increases serum ucOC levels and improves muscle metabolism and whole-body glucose control [10], most likely via increased insulin signaling protein levels within skeletal muscle, and that a decreased ratio of ucOC/tOC correlates with lower muscle strength in older women [19]. However, the effect of acute exercise on ucOC in older adults remains unknown; in particular, the role of ucOC in human myotubes and its association with muscle function parameters (ie, strength and mass) remain unclear.

Exercise causes a series of physiological responses in the bone and skeletal muscle, improving glucose regulation and insulin sensitivity and, importantly, promoting pro-osteogenic factors, including increasing bone formation biomarkers such as osteocalcin [26-31]. Exercise is a known nonpharmacological approach to improving bone health, reducing the risk of osteoporosis, and, importantly, concomitantly improving muscle function [32-35]. Thus, exercise represents an efficacious approach in older persons to reduce age-associated alterations related to sarcopenia, which currently has no available drug treatments. Evidence suggests that various mechanical factors including exercise stimulate the differentiation of mesenchymal stem cells (MSCs) into osteoblasts [36,37]. COP cells circulate within the blood and are MSC-like with osteogenic potential and a precursor for the osteoblastic lineage and potentially osteocalcin [11]. It remains unknown whether exercise, with stimuli promoting pro-osteogenic factors (ie, high load bearing resistance exercise [RE], impact, and jumping exercise), can mitigate the aging process in skeletal muscle and bone through its effect on COP cell levels and therefore osteocalcin [38]. Even a single acute bout of exercise (ie, aerobic exercises [AERs] and REs) elicits positive effects on the bone endocrine and biomarker response and can increase ucOC and insulin sensitivity [9,10,39-42]. However, the exact mechanism by which ucOC influences skeletal muscle function, strength, and metabolism in older adults remains unclear.

Objectives

The primary objective of this study is to determine the change in circulating ucOC following acute exercise interventions between older adults with low and high muscle function (stratified based on leg muscle quality [LMQ]; see the *Lower Limb Maximal Strength and LMQ* section for the LMQ equation). To extend the current knowledge of ucOC in humans, and as an adjunct to this study, we will also perform in vitro experiments on cultured primary myotubes to uncover the mechanistic pathways of action of ucOC. In addition, as a secondary objective, we will aim to quantify the lineage of COP cells before and after exercise, as COP cells can potentially act as a regeneration and antiaging inducible factor and a precursor for osteocalcin.

Hypotheses

We hypothesize that older adults with lower muscle function, compared to those with normal or higher muscle function, will (1) be characterized by lower levels of circulating ucOC and those with lower ucOC will be associated with poorer glucose control and (2) be characterized by abnormal skeletal muscle signaling (muscle hypertrophic or atrophic pathways). Both AER and RE will increase ucOC; however, we hypothesize that this will be to a greater degree in those with lower muscle function. We will test these hypotheses at baseline and after acute exercise. This project has the potential to identify novel biomarkers for interactions between bone and muscle, with implications for future drug targets or clinical interventions and the management of those with or at risk of sarcopenia or reduced muscle function.

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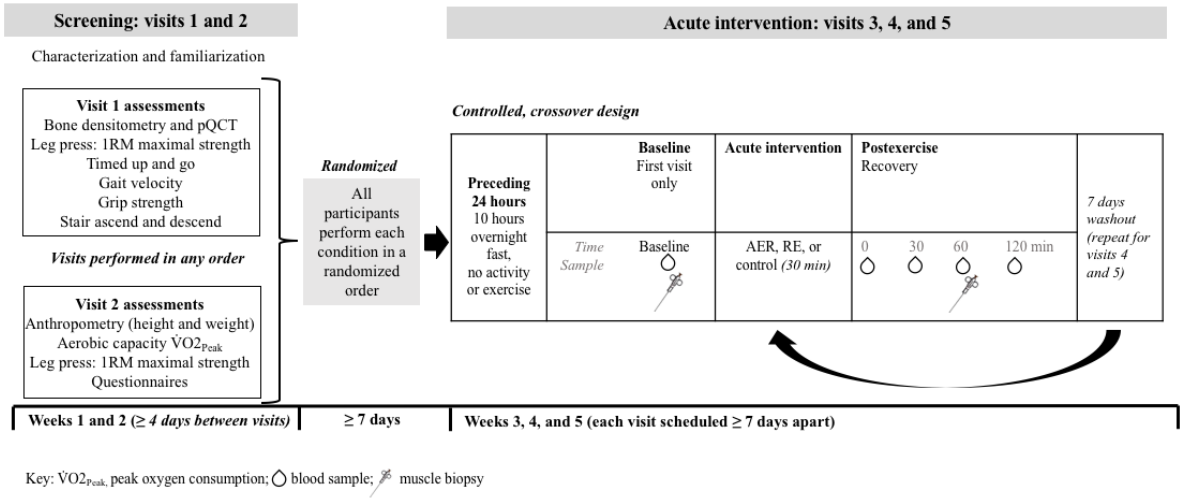
Methods

Design

This study is a randomized controlled crossover trial (Figure 1) approved by the Melbourne Health (MH) Human Research Ethics Committee (reference number: 2017/08) and is registered with the Australian New Zealand Clinical Trials Registry (trial number: ACTRN12618001756213). The trial is a multicenter clinical trial conducted at the Institute for Health and Sport, Victoria University, Melbourne, Victoria, Australia, and the Australian Institute for Musculoskeletal Science (AIMSS) in Western Health, St Albans, Victoria, Australia. The trial will be conducted in accordance with the Helsinki Declaration, and reporting of the study will adhere to the CONSORT (Consolidates Standards of Reporting Trials) guidelines [43,44].

Figure 1. Study design. 1RM: one-repetition maximum; AER: aerobic exercise; pQCT: peripheral quantitative computed tomography; RE: resistance exercise.



Participants

Men and women aged 60 years or older will be recruited. Women will be required to be a minimum of 12 months postmenopause; this is because of the potential alteration in hormones that occur during perimenopause, which can interact with or affect the specific project outcomes of this study. The inclusion and exclusion criteria are listed in Textbox 1. Additional study exclusions will include the inability to provide informed consent independently for safety reasons (particularly as we take some invasive measures) and an inability to understand English, as this may potentially be a safety concern if unable to communicate during visits that include maximal exertion testing and acute exercise bouts.

Textbox 1. Study eligibility.

Inclusion criteria

- Males and females aged 60 years
- Females >12 months postmenopause

Exclusion criteria

- Any fractures within the previous 3 months
- Have begun a new osteoporotic treatment within the previous <3 months or have begun taking antiresorptive medications within the previous <3 months
- Have diabetes mellitus or are taking hyperglycemic medications
- Any hematological, myelodysplastic, or myeloproliferative disorder
- Any bone malignancy
- Taking warfarin of vitamin K supplementation or restriction
- BMI≥40 kg/m^2
- Engagement in a resistance exercise regime for more than 2 sessions per week

Recruitment

Prospective participants will be recruited using advertisement flyers. These will be displayed at Western Health sites (Sunshine and Footscray Hospitals, Victoria, Australia) and provided for use within the general community and other media outlets. Interested participants will self-initiate contact with the research team via email or phone. Those interested will be screened

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against the inclusion and exclusion criteria. Eligible participants will be provided with information for the participants and participant-informed consent forms. A physical examination and an approval to participate in the study will be required from the patients' physician. Please refer to Multimedia Appendix 1 for the schedule of enrollment, interventions, and assessments.

Initial Screening

Summary of Initial Screening

The initial screening will be used for clinical characterization of the volunteers as well as for bone and muscle quantification and quality. It includes 2 separate visits of 3-4 hours' duration (visits 1 and 2; Figure 1), performed in any order and up to 14 days apart. Both visits will be performed in the morning and following an overnight fast. The measures obtained during these visits are explained in detail below.

Bone and Muscle Health

Dual Energy X-Ray Absorptiometry

Body composition and bone mineral density (BMD) will be assessed using a dual energy x-ray absorptiometry (DXA) scanner (Hologic, Horizon A, software version 5.6.0.4). Total BMD as well as the neck of the femur and lumbar spine BMD will be assessed. Lean body mass and fat mass will also be assessed. The DXA scan will ideally be performed in the morning following an overnight fast by experienced personnel. This will be completed by the AIMSS.

Bone Microarchitecture and Fat Infiltration

Peripheral quantitative computed tomography (pQCT; Stratec XCT3000, Stratec Medizintechnik GmbH) will be used to quantify muscle and bone mass, density, and adipose infiltration at the nondominant forearm and foreleg [45,46].

Single 2.5-mm transverse scans will be obtained at 4% and 66% of tibial length (measured form the palpable tip of medial malleolus) and 4% and 66% of the radial length (from the radial condyle), with a voxel size of 0.4 mm. All pQCT scans will be acquired and analyzed by an experienced operator, and the device will be calibrated on the scan date using the manufacturer's phantom. Calf and forearm muscle cross-sectional areas (mm^2) and densities (mg/cm^3) will be determined using the manufacturer's algorithms and software (version 6.2). The calf intramuscular adipose tissue cross-sectional area (cm^2) will be quantified as previously described [47]. Trabecular and cortical bone densities and structure will be assessed at the relevant regions of interest. All imaging will be performed by an appropriate expert (radiographer).

Blood Sample

Quantification of Bone Remodeling and Cardiometabolic Biomarkers

Beta-isomerized C-terminal telopeptide (a bone resorption marker) and procollagen 1 N-terminal propeptide (a bone formation marker) will be quantified using a Roche Hitachi Cobas e602 immunoassay analyzer, according to the manufacturer's guidelines. Hormones (PTH), lipids, glucose and insulin, inflammation markers (C-reactive protein and serum IL-6), and potentially other cardiovascular or health markers will be analyzed according to standard hospital procedures.

Genotyping and Target Genetic Variants Analyses

We will target either candidate gene variants [48,49] or Genome-Wide Association–based variants previously related to skeletal muscle and bone health [50,51]. Genomic DNA will be extracted from residual blood samples from Becton Dickinson (BD) Vacutainer EDTA tubes using the MagSep Blood gDNA kit (0030 451.00, Eppendorf) or GeneJET Genomic Whole Blood DNA Purification Kit (#K0781 Thermo Scientific). Gene variants will be determined using the TaqMan SNP assay (Applied Biosystems, Thermo Fisher Scientific) by QuantStudio 7 Flex (Applied Biosystems, Thermo Fisher Scientific). Genotyping will be replicated in another independent institute, as previously described [52,53], to validate the results.

Muscle Function and Strength

Grip Strength and Gait Velocity

Grip strength will be measured using a hand dynamometer; a result of <20 kg for women and <30 kg for men will identify low muscle strength [54,55]. A 4 m gait velocity assessment will be performed by using the instrumented walkway, which has an acceleration (GAITRite), and by timing with a stopwatch, and reduced physical function will be determined as <80 cm/second. Both the grip strength and gait velocity thresholds noted are accepted as a measurement of sarcopenia [54,55] and will form the definition in this study.

Lower Limb Maximal Strength and LMQ

Participants will perform a one-repetition maximum (1RM) test on a leg press. This will be performed twice, with the first visit serving as familiarization. 1RM is defined as the heaviest weight lifted once with the proper technique and without compensatory movements [56]. Results from this study will guide appropriate prescription for the acute RE session.

LMQ, an estimate of specific force, has been shown to decrease with age and is described as the amount of force a muscle group can produce per unit of muscle mass [57]. We will calculate LMQ as follows:

LMQ = leg strength (kg)/(left leg lean mass [kg] + right leg lean mass [kg]) **(1)** [58].

Leg strength will be defined as the participants' 1RM, and leg lean mass will be obtained from the DXA assessment.

Physical Performance Test

Participants will complete a physical performance test (PPT), adapted from Levinger et al [59], and will include 4 functional mobility tasks: (1) a gait velocity assessment (described earlier), (2) timed up and go test, (3) stair climbing power (SCP), and (4) stair descending. All tests will be scored in time (seconds).

The timed up and go test is a simple performance-based assessment that requires minimal equipment, including a standard arm chair (approximately 46 cm), a 3-m walkway with a floor mark, and a stopwatch (time, seconds). It is performed as time (seconds) taken to rise from a seated position, walk 3

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m, turn, walk back to the chair, and then sit. The SCP consists of a rapid ascent of 10 stairs and is calculated as follows:

Power = force × velocity **(2)**

[60]

Velocity is calculated as the vertical distance of the stairs divided by the time it takes to ascend the stairs. Force is calculated as the participants' body weight multiplied by acceleration due to gravity (9.8 m/s^2). The stair descent will be the time to safely descend 10 stairs. The rest between ascent and descent will be 45 seconds. Participants will undergo 4 attempts on each task, and the best time will be recorded for each task. The PPT score will be the sum of the fastest times recorded for each test.

Aerobic Capacity and Vascular Health

Peak oxygen consumption will be assessed on a cycle ergometer with the initial intensity beginning at 10-30 W and increasing by 10-30 $W \times minute^{-1}$ according to participant ability. Participants will be monitored by a 12-lead electrocardiogram (Mortara, X-Scribe II). Oxygen consumption for each 15-second interval will be measured by gas exchange analysis (BreezeEx, version 3.02, Medical Graphics Corporation), with routine calibration of gas concentrations and flow before each test. The test will be terminated according to participants' self-reported fatigue perception reaching a predetermined level (using the Borg scale, ratings of perceived exertion [RPE]=17) or clinical signs or symptoms [61]. Blood pressure will be monitored at baseline, regular intervals (each stage), and post exercise using a manual sphygmomanometer, and heart rate will be monitored via the 12-lead electrocardiogram.

Vascular endothelial function will be assessed by brachial artery flow–mediated dilatation, used in clinical trials as a reproducible method to assess endothelial function [62,63]. Vascular stiffness will be assessed by noninvasive measures of pulse wave velocity (simultaneous comparison of carotid and femoral arterial pulses) and pulse wave analysis (pulsations recorded at the brachial artery to produce central aortic pressure waveforms) using applanation tonometry (SphygmoCor EXCEL system V1, AtCor Medical) [64].

Questionnaires and Lifestyle Behaviors

Physical Activity Log

Participants will complete a lifestyle behavior and physical activity log. This log has been developed for the purpose of this study and will have questions related to average sleep cycles and normal physical activity levels on weekdays versus weekends (stratified into moderate, hard, and very hard activities). The physical activity component includes consideration for activities of daily living and structured exercise, with examples provided.

Dietary Behavior

A 3-day dietary log will be given to participants on their first visit, to be returned on visit 2 for investigators to analyze normal dietary behaviors. Participants are encouraged to eat normally while they are recording (ie, not to adjust food quantities) and are instructed to complete the dietary log on 2 weekdays and 1 weekend day (consecutively). This log also requests a timed record of physical activity behaviors, including the time and intensity, the time at which food and drinks are ingested, and the time and quantity of medications and supplements.

Falls Risk Questionnaire

The Falls Risk for Older People in the Community (FROP-Com) was developed by the National Ageing Research Institute as a modified version of the Falls Risk for Hospitalized Older People for better utility in the community [65]. The FROP-Com is simple, takes only 10-15 minutes to complete, is low cost, requires no equipment, and can be administered by any health professional. It is a comprehensive fall risk assessment, covering 13 risk factors for falls set out in 26 questions with dichotomous or ordinal scoring (from 0 to 3). The overall score is indicative of fall risk, with the total score ranging from 0 to 60, with higher scores indicating greater risk. The tool has demonstrated good reliability and has a moderate capacity to predict falls [65].

Mini Nutritional Assessment Questionnaire

The Mini Nutritional Assessment (MNA) is a widely used tool for assessing nutritional status in older adults. It is simple to administer, low cost, and validated, with high sensitivity, specificity, and reliability. The MNA classifies the interviewee as well nourished (score≥24), at risk of malnutrition (score between 17 and 23.5), or malnourished (score<17). The MNA also correlates with clinical assessments and objective measures, such as albumin, BMI, triceps skinfold, caloric intake, and vitamin status, and low scores are related to the incidence of clinical events and mortality [66-69].

Charlson Comorbidity Index Questionnaire

The Charlson Comorbidity Index (CCI) is a validated measure of 1-year mortality risk and burden of disease and is used in clinical research to understand the influence of comorbidities and predict outcomes [70-72]. In clinical practice, the CCI assists with the stratification of patients into subgroups based on disease severity to assist with targeted models of care and resource allocation. The CCI includes 17 comorbidities (with 2 subgroups for diabetes and liver disease) that are weighted from 1 to 6 for mortality risk and disease severity. These scores are then tallied to form the total CCI score.

Randomization

Following baseline assessments, participants will be randomized into the acute intervention to explore the characteristics of older adults (by sex) with low or high muscle function (Figure 1). Participants will be randomized individually by a researcher external to this project (they will have no contact with the participants before or during the trial). This person will also have no intellectual or personal investment in the study design, data collection, or outcome. The order of the 3 conditions for each participant (AER, RE, or control) will be randomized using a sealed envelope method (block allocation) to prevent carryover effects between conditions. The envelopes will be stored separately in a locked cabinet, and each envelope will contain 3 pieces of paper that will state "AER," "RE," and "CON." These pieces of paper will be folded to reduce their transparency.

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Study Intervention

Acute Intervention

Participants will complete the acute intervention (visit 3, 4, and 5) up to 14 days after completing the screening assessments and will complete the AER, RE, and CON conditions (Textbox 2) in a randomized order (described below). These visits will include blood sampling and optional skeletal muscle biopsies. Participants can elect to have none, 1 (at rest, for a baseline measure), or 4 biopsies (1 at rest for baseline and 1 following each condition). Each testing visit is approximately 3 hours in duration, including the 30-minute intervention (exercise or rest), and visits will be performed approximately 7 days apart, accounting for washout.

Textbox 2. Acute intervention.

Description of interventions

- Aerobic exercise: Performed on the cycle ergometer for 30 minutes at an intensity corresponding to 70%-75% of peak heart rate; this is based on data obtained from the exercise capacity assessment. Intensity will be adjusted every 5 minutes to maintain the desired heart rate range.
- Resistance exercise: The protocol is as we have previously performed [10] and includes 30 minutes of strength and power exercises at intensities corresponding to 70%-75% of the predetermined one-maximal repetition based on the individual's test results. Leg press will be performed as 5 sets of 10 rapidly concentric (as fast as possible) and slow eccentric (4 seconds) repetitions. Recovery between sets and exercise will be 2 minutes. Participants will also perform jumping sequences as 5 sets of 10 jumps (jumping as high as they can 10 times without stopping). Power training is effective to increase muscle strength and bone density and is safe for older adults [73-75].
- Control: This session will include 30 minutes of supine bed rest.

All testing visits will be monitored and supervised by accredited exercise physiologists (AEPs), who will follow the structured protocol as dictated for that particular session (AER, RE, and CON). The AEP will also monitor signs and symptoms in response to exercise training and will record Borg RPE, blood pressure, and heart rate at frequent time points. Any adverse signs and symptoms will be documented, including feelings of fatigue, soreness, light-headedness, and any injuries. Blood sampling and intravenous cannulation will be performed by personnel who are experienced in the technique, and muscle biopsy will be performed by an experienced medical physician.

Control Procedures

For testing visits 3, 4, and 5, participants will arrive at the laboratory between 7 AM and 8 AM following an overnight fast and with abstinence from exercise or reduced general activity (ie, heavy to moderate activities of daily living) in the preceding 24 hours and for all follow-up sessions. These sampling procedures will be followed at all visits to account for circadian or diurnal rhythms [76]. Participants may be requested to abstain from particular medications (eg, aspirin), as advised by the medical doctor, if electing for a muscle biopsy.

To assist with adherence to study protocols, participants will be monitored via regular communication with the study coordinator on the days preceding each study visit. As a general consideration for participation in this study, participants will be encouraged not to alter their current physical activity levels, exercise habits, or dietary intakes for the entirety of the study. Participants are asked to report whether there have been any alterations in medications throughout the study, as we request that all medication interventions are stable for at least more than three months.

Biospecimen Sampling Protocols

On arrival and following supine rest (approximately 15 minutes), a cannula will be inserted into the antecubital vein, and a baseline (resting) blood sample (40 mL; biopsy, if consented) will be obtained. These baseline (resting) samples will be obtained on the first visit only, serving as the baseline for all other visits thereafter. Four additional blood samples following the 30-minute acute intervention will be collected immediately after the intervention (0-minute time point) and at 30, 60, and 120 minutes postintervention (total of 100 mL) to observe changes in tOC, ucOC, COP, and other measures. If elected, a postintervention biopsy will be conducted at the 60 minutes time point. At all time points, blood samples will be collected into EDTA and serum-separating tubes vacutainers for the appropriate collection of serum or plasma. Following 10-minute clotting time, samples will be centrifuged for 10 minutes at 4° C and immediately transferred to long-term storage at −80° C in 2 mL aliquots for later analysis.

Outcome Measures

Primary Outcomes

The primary outcome for this study is the peak change in circulating levels of ucOC from baseline compared with postacute exercise blood sampling time points (0, 30, 60, and 120 minutes) following the 3 acute interventions (AER, RE, and CON) between the low muscle function and high muscle function groups.

Secondary Outcomes

The secondary outcomes for this study are (1) the difference in protein content related to atrophic and hypertrophic protein signaling at baseline between the low muscle function and high muscle function groups and (2) the difference in protein signaling (protein content) from baseline and compared with the postmuscle sampling timepoint (60 minutes) following the 3 acute interventions (AER, RE, and CON) between the low muscle function and high muscle function groups.

Data Collection and Analysis

Quantification of Osteocalcin

Serum tOC will be analyzed as described previously [9,10,19,39]. In brief, tOC will be measured using an automated immunoassay (Elecsys 170; Roche Diagnostics). Serum ucOC will be measured by the same immunoassay after absorption of

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cOC on 5 mg/mL hydroxyl-apatite slurry, following the method described by Gundberg et al [77].

Quantification of COP Cells

COP cell analysis will be performed as described previously [78,79]. In brief, peripheral blood samples (20 mL) will be collected (EDTA tubes) and processed for Ficoll-based gradient separation, and approximately 5×10^6 peripheral blood mononuclear cells (PBMCs) will be obtained. Approximately 1×10^6 PBMCs will be resuspended in fluorescence activated cell sorting (FACS) buffer, followed by a 10-minute blocking with fragment crystallizable receptor blocking reagent (BD Biosciences). Staining will then be performed with a viability marker (30 minutes), followed by washing (×2) with phosphate buffered saline (containing 5% fetal calf serum). Cells will be incubated with mouse antihuman CD45-Pacific Blue, CD3-PerCP, and CD19-APC (40 minutes). Staining of intracellular components will be permeabilized with Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's instructions, followed by incubation with mouse antihuman osteocalcin-phycoerytrhin at 4 °C (40 minutes), and then washed with Perm or wash buffer (×2).

Flow Cytometry

Cells will be analyzed using a BD FACS Canto II. FACS DiVa software will be used to analyze 50,000 total events for each sample and for the fluorescence minus one (FMO) controls. A total of 3 lasers and 8 different photomultiplier tube (PMT) channels will be used for the 6-color staining panel. Two FMOs will contain fluorochromes, except for the one to be controlled for. Compensation beads will be used to set the compensation controls for each fluorochrome. The PMT voltage values for fluorochromes will be set for each cell type based on the compensation controls. Doublet discrimination will be applied, and viability will be assessed by negative staining using the Live/Dead stain. Offline analysis will be performed using Flow Jo analytical software (Treestar).

Gating Strategy

Cells will be gated for size, shape, and granularity using forward and side scatter parameters, as previously described by our group [78,79]. Briefly, serial gating steps will be applied to quantitate cellular populations. First, dead cells will be excluded, and then, a region will be set to encompass lymphocyte-, monocyte-, and granulocyte-enriched areas, followed by doublet discrimination, T cell (CD3) and B cell (CD19) elimination. For COP cells, after gating on live single mononuclear cells (on forward and side scatter plots), the CD45 and osteocalcin double positive cells will be calculated. Cutoff points to assign antigen positivity will be performed against matching FMO controls. The use of FMO significantly increases the sensitivity and specificity of analysis, as they effectively minimize the effect of nonspecific antibody binding and cell-specific autofluorescence. The gating quantification will be performed twice for the accurate quantification of the percentage of COP cells.

Muscle Sampling Protocol and Analyses

Summary of Sampling Procedure

If elected, the muscle samples (1 or 4) will be taken from the vastus lateralis (approximately 150 mg) under local anesthesia (xylocaine 1%) by percutaneous needle biopsy technique, modified to include suction [80]. Excised tissue will be snap frozen in liquid nitrogen and stored at −80° C for later analysis. Proteins involved in muscle degradation and hypertrophy (ie, anabolic and catabolic pathways; ubiquitin-proteasome, autophagy-lysosome, and caspase-3–mediated proteolytic pathways) as well as glucose uptake will be assessed, as described previously [81-84].

Protein Extraction and Western Blotting

All muscle samples (baseline and postintervention samples) will be used to analyze the content and activation of signaling proteins involved in muscle degradation and hypertrophy by using western blotting, as described previously [84-86]. Western blotting is a method commonly used to detect and analyze the abundance and posttranslational modifications (such as phosphorylation) of proteins. Briefly, muscle samples will be homogenized in a radioimmunoprecipitation assay buffer using a TissueLyzer (QIAGEN). Then, proteins in the lysate will be separated based on protein molecular weight via gel electrophoresis. Proteins will be subsequently transferred to a polyvinylidene fluoride membrane where specific proteins can be probed using specific antibodies. Finally, signals generated through electrogenerated chemiluminescence will be detected and analyzed using ChemiDoc Imaging Systems (Bio-Rad Laboratories).

Primary Skeletal Muscle Cell Culture

A portion of the muscle obtained at rest (baseline) will be used for cell culture for future molecular analyses [87]. This will be established according to the method described by Blau and Webster [88] and by Gaster et al [89] and previously detailed by McAinch et al [90]. Briefly, muscle samples (50-100 mg) will be washed, minced, and enzymatically dissociated with trypsin. Cells will be cultured in a coated flask with extracellular matrix, and the growth medium (α-minimal essential medium [α-MEM]+10% fetal bovine serum+0.5% penicillin-streptomycin+0.5% antifungal) will be changed every other day until they reach 80% confluence. Then, satellite cells will be selected using CD56+ magnetic microbeads (Miltenyi Biotec) and transferred to bigger flasks coated with extracellular matrix to increase cell number (up to 4 passages). Once the cells reach 80% confluence, they will be differentiated using a differentiation medium (α-MEM+2% horse serum+0.5% penicillin-streptomycin+0.5% antifungal) for 5-6 days. Before the experimental treatment, cells will be starved for 1 hour in a serum-free medium (α-MEM+0.5% penicillin-streptomycin+0.5% antifungal).

In vitro treatment with ucOC at concentrations of 0 ng/mL, 30 ng/mL, and 100 ng/mL in serum-free medium for 60 minutes and 24 hours in the presence or absence of insulin (100 nM for the last 15 minutes) for the determination of glucose uptake (2-deoxy-D-[3H] glucose) and western blotting will be performed. Analysis of targeted proteins (described above) will

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be performed as described previously [39,91]. The dose response is important because the physiological effect of ucOC in muscles from mice and humans may be different, and the concentrations used are all physiologically relevant.

Participant Retention and Withdrawal

Once a participant is enrolled into the trial, the study coordinator will keep in contact with him or her for the entire study period. We estimate that the dropout rate in this population will be approximately 10%. Participants may withdraw from the study at any given time. The investigators or medical staff may also withdraw participants from the study due to safety or medical concerns.

Statistical Analysis and Determination of Sample Size

The primary endpoint for this study is the change in ucOC levels from baseline to the peak, postintervention sampling time point. The analysis will include a comparison of changes in ucOC levels in response to each intervention from baseline to postintervention sampling time points (0, 30, 60, and 120 minutes) between the low muscle function and normal or high muscle function groups, by using repeated measures analysis of variance (ANOVA). Comparisons of multiple means will be examined using a 2-factor (exercise type×time point) repeated measures ANOVA. For all significant interaction and main effects, a priori comparisons of means (baseline vs all postexercise time points) will be conducted using the Fisher least significant difference test ($P<.05$). Multivariable regression models will be used to determine associations between selected measurements, adjusting for BMI and sex. Data will be analyzed using the Statistical Package for the Social Sciences, version 22 (SPSS Inc), and statistical significance will be declared at $P<.05$.

In 10 postmenopausal women, we previously reported that the change in ucOC levels following exercise is approximately 9% [39]. We will recruit 54 participants (equal number of men and women) who will be dichotomized as low muscle function versus high muscle function (27 per group). After adjusting for a loss to follow-up rate of 10% (5.4/54), this sample size will be large enough to detect an estimated 4% difference in changes in ucOC levels (SD 6%) between groups with a type I error rate of 5%, type II error rate of 20%, and power of >80% (G*Power 3.1.9.2 for Windows) [92].

Data Monitoring

Data Management and Monitoring

Details of the procedures for data management have been reviewed and approved by the MH Human Research Ethics Committee and can be located via study reference, 2017/08. A trial management group (TMG) has been formed to manage potential risks and for structured oversight of the trial [93]. The type of oversight that this TMG will provide includes regular meetings to review individual safety reports and data relating to quality, protocol adherence, and participant retention rates. The TMG committee will include the principal investigator; individuals responsible for the daily running of the trial, including the trial coordinator; and an appointed independent member.

All electronic data will be stored on password-protected computers. Hard copies of any data will be kept in a locked filing cabinet in a secure office. All data collection tools and questionnaire data will be deidentified.

Harms

All adverse events associated with the study, or occurring during study participation, will be recorded. All adverse events will be reported to the TMG and ethics committees (MH and Victoria University) with strategies to reduce the risk for future events. The ethics committees have the power to pause or even stop the research in the case of a severe adverse event. The study personnel will monitor the clinical signs and symptoms of dyspnea, shortness of breath, nausea, faint-headedness and light-headedness, signs of inflammation, or infection throughout the study period.

Auditing

The TMG will meet and run an internal audit of the trial at regular intervals annually. The principal investigator, IL, is responsible for the overall conduct and preservation of the integrity of this trial and has extensive experience as a lead research investigator in numerous human clinical trials.

Ethics and Dissemination

This study and its protocols and data collection tools have been reviewed and approved by the MH Human Research Ethics Committee (ref approval number: HREC/17/MH/335; local project number: 2017/208), and local ethical approval has been confirmed at Victoria University as a mirror approval of MH. Any modifications to the study objectives, procedures, protocols, data collection tools, and study personnel will require a formal amendment from the ethics committee. All protocols related to consenting procedures, data collection and access to participant data, procedures for maintenance of participant confidentiality, and plans for dissemination of study results can be found in the reviewed and approved documents of trial reference 2017/208.

Results

The trial is active, with participant recruitment and intervention delivery currently ongoing (MH Human Research Ethics Committee ref approval number: HREC/17/MH/335; Western Health Sunshine Hospital local project number: 2017/208; protocol version number: 6 25/06/2018). Recruitment for this trial began in February 2018, and 38 participants have completed the study as of May 26, 2019. The results of this study will be published throughout the trial, and the main study findings are expected to be published by June 2021.

Discussion

Previous research suggests that crosstalk exists between the skeleton and skeletal muscle; however, this crosstalk has not been fully described or clearly elucidated, particularly in humans. In addition, a lack of physical activity accelerates the widespread cellular and molecular changes induced by aging, resulting in an increased prevalence of many chronic diseases [94]. Detecting the age-related conditions associated with inactivity and early intervention are essential for reducing the

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economic burden of aging on the health care systems worldwide. The development of affordable and universally accessible ways to prevent chronic disorders, such as tailored exercise programs, in combination with the development of robust blood biomarkers, will considerably improve the ability to predict and detect chronic diseases and reduce the health and economic burden caused by aging in a cost-effective manner [95].

This project is designed to uncover a novel crosstalk pathway between bone and muscle in older adults via ucOC. Current evidence from predominately cross-sectional studies suggests that osteocalcin, via ucOC, in humans may be associated with muscle function [10,19]. Evidence from animal and preclinical studies is encouraging, indicating a promising role for ucOC in improving muscle metabolism and function [16,17,25]. However, the roles of ucOC in humans and its relationship with muscle function and metabolism remain unknown. Evidence suggests that COP cells have a dynamic capacity to mobilize to sites of fracture repair and have the capacity to be upregulated under varying pathological or physiological bone forming processes, such as puberty and fracture [79,96-102]. However, it is unknown whether exercise stimulus, with osteogenic capacity, can increase the COP cell population and upregulate tOC and therefore ucOC.

The proposed project aims to overcome this gap by characterizing ucOC levels in older adults with a spectrum of muscle functions and in response to an acute exercise intervention. Importantly, we will investigate this bone and muscle crosstalk by determining the associations between the parameters of muscle function (ie, muscle signaling, muscle mass, and muscle strength) and ucOC. In the future, we plan to directly assess this association at a cellular level in human primary myotubes (those prepared in this study) to determine the direct effects of ucOC on muscle protein signaling and glucose uptake. The results of this study will provide a greater understanding of skeletal muscle metabolism and the crosstalk between muscle and bone in the older adult population. We aim to establish ucOC as a biomarker for muscle function and bone and muscle crosstalk in older adults to target potential mechanisms for future therapeutic studies. We also aim to advance the development of personalized clinical exercise guidelines for sarcopenia and other musculoskeletal conditions.

Acknowledgments

This work is supported by research funds of the primary investigators, IL and GD, and the PhD (Doctor of Philosophy) funds of CS. This trial has been awarded the Tom Penrose Community Service Award from Exercise and Sport Science Australia, a seed grant from AIMSS, and a Diabetes Australia Research Program Grant from Diabetes Australia. The grants awarded to this trial did not have any role in or contribution to the design of the study and the collection, analysis, or interpretation of the data; the grants awarded to this trial will not contribute to the writing of any associated manuscripts.

Authors' Contributions

CS participated in the trial design, coordination and intervention delivery of the trial, and collection of data at Victoria University and Western Health and drafted the manuscript. IL participated in the design of the study, contributed to data collection at Victoria University and Western Health, and helped draft the manuscript. GD participated in the study design, was involved in the trial as an advising medical physician, and reviewed the manuscript. The remaining authors, XL, DS, TB, AA, AM, MW, EB, and NE, contributed or specialized in specific expert techniques; and CS, IL, GD, XL, DS, TB, AA, AM, MW, EB, and NE read and approved the final manuscript.

Conflicts of Interest

None declared.

Multimedia Appendix 1

Schedule of enrollment, interventions, and assessments.
[DOCX File , 20 KB-Multimedia Appendix 1]

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Abbreviations

1RM: one-repetition maximum
AEP: accredited exercise physiologist
AER: aerobic exercise
AIMSS: Australian Institute for Musculoskeletal Science
ANOVA: analysis of variance
BD: Becton Dickinson
BMD: bone mineral density
CCI: Charlson Comorbidity Index
cOC: γ-carboxylated osteocalcin
COP: circulating osteoprogenitor
DXA: dual energy x-ray absorptiometry
FACS: fluorescence activated cell sorting
FMO: fluorescence minus one
FROP-Com: Falls Risk for Older People in the Community
IL-6: interleukin-6
LMQ: leg muscle quality
MH: Melbourne Health
MNA: Mini Nutritional Assessment
MSC: mesenchymal stem cell
PBMC: peripheral blood mononuclear cell
PMT: photomultiplier tube
PPT: physical performance test
pQCT: peripheral quantitative computed tomography
PTH: parathyroid hormone
RE: resistance exercise
RPE: ratings of perceived exertion
SCP: stair climbing power
TMG: trial management group
tOC: total osteocalcin
ucOC: undercarboxylated osteocalcin
α-MEM: α-minimal essential medium

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Edited by G Eysenbach; submitted 18.03.20; peer-reviewed by L Santacroce, A Sule, S Rassi; comments to author 01.09.20; revised version received 29.11.20; accepted 01.12.20; published 09.04.21

Please cite as:
Smith C, Lin X, Scott D, Brennan-Speranza TC, Al Saedi A, Moreno-Asso A, Woessner M, Bani Hassan E, Eynon N, Duque G, Levinger I
Uncovering the Bone-Muscle Interaction and Its Implications for the Health and Function of Older Adults (the Wellderly Project): Protocol for a Randomized Controlled Crossover Trial
JMIR Res Protoc 2021;10(4):e18777
URL: https://www.researchprotocols.org/2021/4/e18777
doi: 10.2196/18777
PMID:

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