Musculoskeletal and vascular ageing:

Points of intersection

By

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

Doctor of Philosophy
Monash University, 2018

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Si se cree y se trabaja, se puede

- Diego Simeone

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General Declaration for Thesis Including Published Works

Declaration for thesis based on conjointly published or unpublished work

Monash University

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations, the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis. This thesis includes eight manuscripts in total including seven published in peer-reviewed journals comprising four original research papers and three systematic reviews and meta-analyses representing original epidemiological research. The other manuscript accepted for publication is a narrative review. The core theme of my thesis focuses on the relationship between components of musculoskeletal health and vascular disease including investigating the effects of bone-important minerals and hormones on various vascular end-points. The ideas, development and writing of papers in this there were the principal responsibility of myself, the candidate, working with the Bone & Muscle Health

Research Group at Monash University, under the supervision of Professor Peter R. Ebeling AO and Dr David Scott.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges the input within a team-based environment.

For Chapters 1 to 5, the following outlines my contribution to each manuscript:
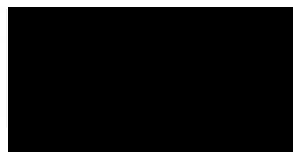
Chapter	Publication title	Status	Nature and % of candidate contribution	Co-author name(s), nature and % of contribution	Co-author Monash student (Y/N)
1.2	Exploring the links between common diseases of ageing – osteoporosis, sarcopenia and vascular calcification	Accepted	80% Primary responsibility for concept and design, literature search, article analysis and interpretation and manuscript drafting	Peter R. Ebeling: (10%) Input into manuscript	No
				David Scott: (10%) Input into manuscript and interpretation	No
2.2	Lower muscle tissue is associated with higher pulse wave velocity: A systematic review and meta-analysis of observational study data.	Published	70% Primary responsibility for concept and design, literature search and screening, data extraction and quality appraisal, data analysis and interpretation and manuscript drafting	Nazmul Md Karim: (10%) Data extraction and quality appraisal, reviewed statistical analysis	No
				Velandai Srikanth: (5%) Input into manuscript	No
				Peter R. Ebeling: (5%) Input into manuscript	No
				David Scott: (10%) Input into manuscript and interpretation	No
2.3	Low Relative Lean Mass is Associated with Increased Likelihood of Abdominal Aortic Calcification in Community-Dwelling Older Australians.	Published	60% Primary responsibility for concept and design, data acquisition and analysis, interpretation and manuscript drafting	David Scott: 20% Input into manuscript and interpretation	No
				Remaining authors	
				Belal Khan: 5% Calcification measurement	No
				Nayeb Khan: 5% Calcification measurement	No
				Allison Hodge: 5% Input into manuscript	No
				Dallas English: 5% Input into manuscript	No
				Graham G. Giles: 5% Input into manuscript	No
				Peter R. Ebeling: 5% Input into manuscript and interpretation	No
2.4	Aortic calcification is associated with five-year decline in handgrip	Published	60% Primary responsibility for concept and design, data acquisition and analysis,	Joshua Lewis: (20%) Input into manuscript and interpretation	No
				Remaining authors	
				Douglas P. Kiel: 2% Input into manuscript	No

	strength in older women		interpretation and manuscript drafting	John T. Schousboe: 2% Input into manuscript	No
				David Scott: 2% Input into manuscript	No
				Peter R. Ebeling: 2% Input into manuscript	No
				Richard L. Prince: 2% Input into manuscript, data custodian	No
3.2	Associations between hip bone mineral density, aortic calcification and cardiac workload in community-dwelling older Australians.	Published	70% Primary responsibility for concept and design, data acquisition and analysis, interpretation and manuscript drafting	David Scott: 15% Input into manuscript and interpretation	No
				Remaining authors	
				Allison Hodge: 5% Input into manuscript	No
				Dallas English: 2.5% Input into manuscript	No
				Graham Giles: 2.5% Input into manuscript	No
				Peter R. Ebeling 5% Input into manuscript and interpretation	No
4.2	Effect of vitamin D supplementation on measures of arterial stiffness: a systematic review and meta-analysis of randomized controlled trials.	Published	80% Primary responsibility for concept and design, literature search and screening, data extraction and quality appraisal, data analysis and interpretation and manuscript drafting	David Scott: (5%) Input into manuscript	No
				Velandai Srikanth: (10%) Input into manuscript and interpretation	No
				Peter R. Ebeling: (5%) Input into manuscript	No
4.3	Effects of vitamin D supplementation on inflammatory markers in heart failure: a systematic review and meta-analysis of randomised controlled trials	Published	45% (equal contribution as joint first author) Primary responsibility for concept and design, literature search and screening, data extraction and quality appraisal, data analysis and interpretation and manuscript drafting	Aya Mousa: (45%, equal contribution as joint first author), data extraction and quality appraisal; data analysis and interpretation and manuscript drafting	Yes
				Remaining authors	
				David Scott: 2.5% Input into manuscript	No
				Peter R. Ebeling: 2.5% Input into manuscript	No
				Barbora de Courten: 5% Input into manuscript	No
4.4	High calcium intake in men, not women, is associated with increased all-cause mortality risk: Melbourne	Published	60% Primary responsibility for concept and design, data acquisition and analysis, interpretation and manuscript drafting	Bo Abrahamsen: (20%) Input into manuscript, data interpretation, clinical interpretation, statistical advice	No
				Remaining authors	

	Collaborative Cohort Study			David Scott: 6% Input into manuscript and interpretation	No
				Belal Khan: 2% Statistical advice	No
				Allison Hodge: 3% Input into manuscript	No
				Dallas English: 2% Input into manuscript	No
				Graham Giles: 2% Input into manuscript	No
				Peter R. Ebeling: 5% Input into manuscript, data interpretation, clinical interpretation	No

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

Student signature: Date: 22 November 2018

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author, I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: Date: 22 November 2018

Synopsis

Vascular disease may be evidenced by manifest structural features in blood vessels such as ectopic calcification of the vascular wall or through surrogate markers such as increased arterial stiffness. Such surrogate markers of vascular ageing are both robust and consistently associated with increased cardiovascular and all-cause mortality and have been shown to improve cardiovascular risk prediction [1, 2]. Indeed, cardiovascular events that have a vascular origin such as myocardial infarction is one of the leading causes of death in Australia and globally [3]. Calcium deposition on the vascular wall is one of the most distinct features of advanced vascular disease. Calcification is visible on lateral spine radiographs or DXA[4]. Calcification in the aorta, particularly in the more distal abdominal aorta is a robust indicator of cardiovascular disease, events and mortality [5]. Functional indicators of structural/manifest vascular disease such as increased arterial stiffness is also predictive of cardiovascular disease, events and mortality [6].

There is growing appreciation of the importance of the musculoskeletal system to the vasculature. Due to consistent observations of falls and fractures being highly prevalent in individuals with cardiovascular disease, and vice-versa, understanding these links is important as vascular disease may only manifest when disease is at a more advanced stage and likely to appear in older age [7, 8]. The effects of muscle and bone loss may become more apparent, or at least become clinically important

(such as difficulties with activities of daily living) at an earlier age and thus offer an on opportunity for intervention and prevent the onset of structural vascular disease. Biologically, muscle and bone share an intimate relationship given their anatomical proximity as well as a strong biomechanical relationship. Muscle and bone are considered to exist as a physiological "unit" and communicate closely through a number of muscle and bone derived cytokines [9, 10]. Epidemiological evidence has revealed an inverse relationship between bone mass and vascular calcification to support decades of observations of the coincidence of osteoporosis and cardiovascular disease [11]. Recently, the loss of muscle mass and function (sarcopenia) has gained importance as a clinically significant disease but evidence for the contribution of muscle mass and function to vascular disease is limited [12]. Furthermore, hormones and minerals important for bone and muscle function including calcium and vitamin D are known to also influence the vasculature [13, 14]. Their role in ameliorating underlying vascular diseases such as impaired arterial function and other cardio-protective effects have yet to be established [15–18].

The theme of this thesis aims to further an understanding of the importance of musculoskeletal health (including micronutrients involved in the musculoskeletal system) to vascular stiffness and calcification.

This theme will be explored in three chapters that separately investigate: (i) the relationship between the musculature and the vasculature; (ii) the relationship

between the skeleton and the vasculature; and (iii) effects of micronutrients on vascular disease. In these investigative chapters a variety of clinical research methods will be employed including evidence synthesis by way of systematic review and meta-analysis which represents original epidemiological research and observational research including cross-sectional analyses and longitudinal analysis. These analyses each employ distinct analytical methods.

This programme of research of this thesis begins with a meta-analysis examining the association between muscle mass and arterial stiffness. Whilst there are consistent observations in the literature that lower muscle mass is associated with increased arterial stiffness [19, 20], no data exists quantifying this relationship to understand the potential contribution of muscle loss to arterial stiffness. Next, in a cross-sectional study, the association between muscle mass and aortic calcification will be examined representing a more robust investigation of a potential muscle-vascular axis. In a longitudinal analysis, the effect of aortic calcification on functional decline in older women will then be explored. These studies will represent a thorough investigation of a potential muscle-vascular axis by examining both muscle mass and function and direct (calcification) and indirect (arterial stiffness) measures of vascular disease.

The thesis progresses from muscle onto bone and its relationship with vascular disease. Most observational evidence exploring the bone-vascular axis has focused

on the association of bone loss with increased vascular calcification[21]. However, little attention has been paid to the effects of vascular calcification on heart function and whether these potentially adverse effects are seen more in those with low bone mass. This is particularly important as other observational studies have already determined that heart structure and function is abnormal in those with low bone density [22]. A mediation analysis will investigate direct associations of bone density with heart function and the potential contribution of aortic calcification to this relationship.

This thesis ends with examination of the effects of calcium consumption and vitamin D supplementation (both important for musculoskeletal health) on various vascular end-points. There is much controversy about the clinical usefulness of vitamin D supplementation in a variety of settings [23]. This thesis will delve into this area of research by investigating the direct effects of vitamin D supplementation on the augmentation index and pulse wave velocity in a meta-analysis. In another meta-analysis, the effectiveness of vitamin D supplementation to resolve underlying inflammation (a potential cause of vascular disease) in the context of heart failure is explored. Controversy also exists as to the risks or benefits of calcium consumption and what amounts are appropriate to recommend [24]. The final study in this thesis will explore the sex-specific effects of dietary calcium intake on cardiovascular mortality.

Overall, this thesis' theme of research investigates relationships between various aspects of the musculoskeletal system (muscle mass, muscle function and bone mass) with surrogate (arterial stiffness) and robust (calcification) markers of vascular disease. Also, this thesis investigates potential clinically important effects of micronutrients important to musculoskeletal health (vitamin D and calcium) on cardiovascular mortality and the underlying mechanisms of cardiovascular disease. In total, this thesis represents a broad investigation of the clinical and biological relationship between two important body systems (musculoskeletal and vascular).

Personal and Professional Reflection on the PhD Candidature

This thesis contains eight manuscripts comprising four chapters in total. Seven of the manuscripts have been published and the remaining one, an invited review, accepted for publication. Included as appendices are nine other manuscripts that were completed during candidature. These manuscripts are not directly relevant to the themes of this thesis but represent my contribution and collaboration with other teams, development of other research skills, general engagement and profile raising with the literature.

Prior to my candidature I had been working alongside a research team at the Australian National University (ANU). With this team, I had the opportunity to work on clinical projects that involved meeting and examining real people and I was also granted responsibilities in handling the publication of our work. This fully motivated me to pursue further research opportunities and after a chance meeting at the 2014 annual scientific meeting of the Endocrine Society of Australia with Professor Peter Ebeling, my supervisors at ANU encouraged me to approach Professor Ebeling about undertaking a PhD.

The rest as they say is history. Throughout my candidature I have matured as a researcher immensely. Professionally, I wanted to develop tangible skills that I could take into my future academic career. Being involved in a randomised controlled clinical trial comparing vitamin D and exercise to exercise alone in a cohort of overweight/obese older adults, I gained experience and now believe I am highly competent in, many aspects of a clinical trial. Specifically, I had primary

responsibility for ethics applications, amendments and progress reporting, adverse event reporting; advertisement and recruitment of participants; screening of participants; scheduling of appointments, preparation of documentation; liaising with hospital departments including Pathology and Admissions; securing a commercial agreement with a pharmaceutical company for the supply of a study drug (vitamin D and placebos); achieving competency in the operation, analysis and interpretation of dual-energy x-ray absorptiometry and peripheral quantitative computed tomography; achieving competency in the operation, analysis and interpretation of pulse wave analysis; examination of participants in the trial including anthropometric and physical function tests; instruction and supervision of the exercise intervention; data collection, entry, cleaning, database management; data analysis including the learning of coding to use the software package *Stata*; writing and publishing manuscripts in peer-reviewed journals and presentation of results at academic conferences. An important professional skill I have strengthened (out of necessity) during my candidature has been flexibility. My project evolved from drawing conclusions from interventional studies to observational and epidemiological studies. Having this ability to achieve outcomes in a changing environment is an important attribute for a researcher and my research team provided the platform for this. This is especially important as one cannot predict the eventual direction of the research from the outset.

I have been fortunate to have been involved with a professional Taskforce of the American Society for Bone and Mineral Research reporting on the safety and

efficacy of vertebral augmentation techniques to treat osteoporotic compression fractures in the spine. Also, my achievements and progress throughout my candidature have been recognised by the Australia New Zealand Bone and Mineral Society as I was selected as an inaugural committee member of the Early Career Investigator Committee within the Society. My crowning achievement though has been securing the Christine & T.J. Martin Research Travel Grant ($12,900) which is the Society's most prestigious award. In total, throughout my candidature I attracted over $44,000 of combined grants and prizes and this history of success should bode well for the next stage in my career transitioning from student to professional.

Personally, my PhD has considerably enhanced my communication, collaborative and problem solving/creative thinking skills. Overall, my PhD has proved to be much more than a scientific endeavour. I cultivated relationships with eminent international researchers at conferences and also with participants for the RCT as screening was primarily phone-based, I genuinely enjoyed chatting to prospective participants over the phone rather than just giving out information in a bland manner and I believe this helped to improve enrolment success.

The body of work in this thesis demonstrates my progression as a researcher and development of skills in clinical research.

Relationship between the muscular-skeletal-vascular systems in ageing: narrative review

Despite much literature exploring the links between muscle and bone, bone and minerals, bone and cardiovascular disease there is little comprehensive exploration into the relationships (clinical and molecular) between these three body systems: muscular-skeletal-vascular. Vascular disease accounts for one-quarter of non-coronary deaths in Australia and thus understanding vascular disease in the context of other common features of ageing is underappreciated. Chapter 1.2 is a narrative (invited) review synthesising the literature in this area. Particular attention is focussed on calcification and arterial stiffness and in understanding the potential therapeutic benefit of calcium and vitamin D on vascular endpoints.

Relationship between muscle mass and function with vascular disease: observational research and evidence synthesis

After exploring the sarcopenia literature, I recognised there appeared to be consistent conclusions in the few studies examining the relationship between muscle mass and arterial stiffness. Thus, in Chapter 2.2, I synthesised this work into a meta-analysis. This work prompted exploration of the association between sarcopenia and aortic calcification which is presented in Chapter 2.3. This original research was a secondary analysis of a subset of individuals from the Melbourne Collaborative Cohort Study who had radiographs for aortic calcification measurements. In Chapter 2.4, I provide another secondary analysis of physical function data from a

randomised controlled trial of calcium supplementation in post-menopausal women. This work was done collaboratively with Dr Joshua Lewis (Edith Cowan University) and Prof Richard Prince (University of Western Australia) for which I had to secure data sharing agreements and design the analysis *a priori*. This last manuscript is important because it completes a full suite of examination of muscle mass (2.2, 2.3) and function (2.4) and its relationship with vascular disease including surrogate markers (2.2) and robust markers (2.3, 2.4).

Relationship between bone density and vascular disease: observational research and evidence synthesis

In Chapter 3, I explore the relationship between bone health and aortic calcification. For some time, our orthodox understanding has been that there is an inverse relationship between bone and the extent of vascular calcification. Therefore, in my investigation I examined what effect aortic calcification had on heart function which has not been previously done. Previously, it has been assumed that vascular calcification affects heart function but my work in Chapter 3.2 was the first to investigate this in healthy older people and connect it to bone health. Indeed, I learnt a statistical technique called mediation analysis to confirm a biological pathway between bone-vascular calcification-heart function.

<u>Effects of micronutrients on vascular disease: evidence synthesis and observational research</u>

Chapter 4 provides studies examining the effect of vitamin D supplementation on arterial stiffness (Chapter 4.2) and systemic inflammation in heart failure (Chapter 4.3) as well as the effect of calcium intake on all-cause mortality and cardiovascular disease (Chapter 4.4). Chapters 4.2 and 4.3 are systematic reviews and meta-analyses on the effects of vitamin D supplementation in various vascular contexts. The analysis on arterial stiffness, at the time of submission of this thesis, had attracted 37 citations and the analysis in heart failure was a project completed collaboratively with another PhD student from Monash University (Dr. Aya Mousa). For the analysis concerning heart failure, the protocol had been developed *a priori* and it was my approach to this group with a proposal to investigate cardiovascular end-points that lead to the formation of the collaboration. The final chapter (4.4) was a project lead by myself and I was guided by international expert on musculoskeletal epidemiology Professor Bo Abrahamsen from the University of Southern Denmark. To do this analysis, I greatly enhanced my statistical coding skills employing specific techniques such as Cox regression and restricted cubic spline curves.

Acknowledgements

I wish to express immense gratitude to my academic supervisors Professor Peter Ebeling and Doctor David Scott. You have been more than supervisors, you were mentors. To Peter, I wish to personally thank you for offering me the opportunity to work alongside you and accepting me into the team. You have continually inspired me, placed your trust in me for important work and allowed me to flourish through exciting and sometimes international opportunities. I am pleased to have also built a friendship during this time and am proud to have your mentorship. I hope our association continues for many years. To David, you have taught me so much about clinical research, working independently and collaboratively and importantly about my reasons for doing things. You have that 'knack' for asking the right question, usually to the very thing I have forgotten to consider. Thank you for always making the time for me. To the members of the Bone & Muscle Health Research Group: Mrs Ruth Fantozzi, Dr Ayşe Zengin, Mr Jakub Mesinovic, Mx Catherine Shore-Lorenti, Dr Jasna Aleksova (and honorary member Dr Sabashini Ramchand) thank you all for your support, banter, food talk and the collegial environment created. You have all made these last few years most memorable. I want to especially acknowledge Doctor Hanh Nguyen who was there just after I started. Talking with you professionally and personally helped me gather my thoughts many times. To Doctor Cecilia Xu, without you I would not have achieved half the things I did during your time with us, you are an excellent companion. Finally, to the soon to be Doctor Lachlan McMillan, I was very pleased to get another lad in the team. You

have been a loyal mate, my go-to person with problems, ideas, footy kicking breaks or just general nonsense and an outstanding colleague to have. You have made my PhD journey more enjoyable and one of the best parts about it. I want to acknowledge the mentorship I received from Doctor Joshua Lewis (Edith Cowan University). Thank you for providing me with collaborative opportunities and giving me my first taste of "swimming with the big boys". I thank Professor Velandai Srikanth loaning of his equipment and general advice on vascular topics. I thank Professor Bo Abrahamsen for his mentorship and collaboration during my stint in Odense, Denmark. Personally, I could not have made it through these last few years without the support and encouragement of my friends and family. To my fellow amigo (Doctor) Aaron Giles, thank you for your advice and the good times you always bring. To my mum and dad, you have given me the world and invested so much of your life in me. The rest of my life will be dedicated to seeing those investments returned in every way. Marissa, although you live so far away you are never far from my thoughts and I know I can rely on you to understand my struggles when I need it. Most importantly, I want to give my most sincere gratitude to my partner Ms Grace Mickleburgh. We have spent the last few years apart, but I like to think we have grown closer together for all that has occurred. You have really been my other half in every important moment of the past few years. There is nothing more profound that I can say other than I could not have done this let alone anything else without you. I want to take the next big step in my life together with you.

Hasta la victoria, ¡siempre!

List of abbreviations

AAC – abdominal aortic calcification
ACS – aortic calcification score
AIx – augmentation index
ALM – appendicular lean mass
BMC – bone mineral content
BMD – bone mineral density
BMI – body mass index
CRP – C-reactive protein
CT – computed tomography
CVD – cardiovascular disease
DBP – diastolic blood pressure
DXA – dual-energy x-ray absorptiometry
HR – hazard ratio
HR-pQCT – high-resolution peripheral quantitative computed tomography
IL - interleukin
IMAT – intra-/inter-muscular adipose tissue
IU – international unit
Ln – Natural logarithm
MCCS – Melbourne Collaborative Cohort Study
OR – odds ratio
pQCT – peripheral quantitative computed tomography
PWV – pulse wave velocity
RCT – randomised controlled trial
RPP – rate pressure product
RR – risk ratio
SBP – systolic blood pressure
SD – standard deviation
SMD – standardised mean difference
SMC – Swedish Mammography Cohort
TNF – tumour necrosis factor
USA – United States of America
UK – United Kingdom of Great Britain and Northern Ireland
Wnt – Wingless related integration site
25(OH)D/25OHD – 25-hydroxyvitamin D
95% CI – 95 percent confidence interval

List of Publications

- Rodríguez AJ, Scott D, Ebeling PR "Exploring the links between common diseases of ageing – osteoporosis, sarcopenia and vascular calcification" Clin Rev Bone Miner Metab (Accepted) [Chapter 1.2]

- Rodríguez AJ, Karim Nazmul MD, Srikanth V, Ebeling PR, Scott D "Lower muscle tissue is associated with higher pulse wave velocity: a systematic review and meta-analysis" Clin. Exp. Pharmacol. Physiol. (2017) Oct;44(10):980-992. doi: 10.1111/1440-1681.12805. [Chapter 2.2]

- Rodríguez AJ, Khan B, Khan N, Scott D, Hodge A, English DR, Giles GG, Ebeling PR. "Low relative lean mass is associated with increased likelihood of abdominal aortic calcification in community-dwelling older Australians." Calcif. Tissue Int. (2016) Oct;99(4):340-9. doi: 10.1007/s00223-016-0157-z [Chapter 2.3]

- Rodríguez AJ, Lewis JR, Scott DS, et al. Aortic Calcification is Associated with Five-Year Decline in Handgrip Strength in Older Women. Calcif Tissue Int (2018) 103:589–598 doi: 10.1007/s00223-018-0458-5 [Chapter 2.4]

- Rodríguez AJ, Scott D, Hodge A, English DR, Giles GG, Ebeling PR "Associations between hip bone mineral density, aortic calcification and cardiac workload in community-dwelling older Australians." Osteoporosis Int. (2017) Jul;28(7):2239-2245. doi: 10.1007/s00198-017-4024-1 [Chapter 3.2]

- Rodríguez AJ, Scott D, Srikanth V, Ebeling PR "Effect of vitamin D supplementation on measures of arterial stiffness: a systematic review and meta-analysis of randomised controlled trials" Clin Endocrinol (Oxf) (2016) May;84(5):645-657. doi: 10.1111/cen.13031. [Chapter 4.2]

- Rodríguez AJ*, Mousa AM*, Scott D, Ebeling PR, de Courten B "Effects of vitamin D supplementation on inflammatory markers in heart failure: a systematic review and meta-analysis of randomised controlled trials" Sci Rep (2018) Jan 18;8(1);1169. Doi:10.1038/s41598-018-19708-0 [Chapter 4.3]

- Rodríguez AJ, Scott D, Khan B, Khan N, Hodge H, English DR, Giles GG, Abrahamsen B & Peter R. Ebeling "Dietary calcium intake is associated with increased mortality and cardiovascular risk in men but not women" Arch Osteoporos (2018) Sept 21; 13:101. doi: 10.1007/s11657-018-0518-5 [Chapter 4.4]

*Asterisk indicates joint first author

List of Conference Presentations

- ECTS 2018 "Evidence supporting a vascular disease-muscle function relationship" Rodríguez AJ, Lewis JR, Scott D, Kiel KP, Schousboe JT, Ebeling PR, Prince RL. Oral abstract #PO25. Valencia, Spain

- ASBMR 2018 "High dietary calcium intake in men, not women, are associated with increased all-cause mortality: the Melbourne Collaborative Cohort Study" Rodriguez AJ, Scott D, Khan B, Hodge A, English D, Giles G, Abrahamsen B, Ebeling PR. Plenary Poster #0829. Montreal, Canada

- ANZBMS 2018 "Aortic calcification is associated with accelerated five-year declines in grip strength in older women" Rodriguez AJ, Lewis JR, Scott D, Kiel KP, Schousboe JT, Ebeling PR, Prince R. Poster #27. Queenstown, New Zealand

- ANZBMS 2018 "Prevalence of cardiovascular risk factors in a population-based cohort of Danish bisphosphonate users: The Odense Bisphosphonate Safety Study" Rodriguez AJ, Ernst MT, Nybo M, Prieto-Alhambra D, Hermann P, Abrahamsen B. Poster #81. Queenstown, New Zealand

- WCO-IOF-ESCEO 2018 "Increased mortality risk in men with high dietary calcium intake but in women" Rodríguez AJ, Scott D, Khan B, Hodge A, English D, Giles G, Ebeling PR Poster Oral #P188 Krakow, Poland

- BRS 2018 "Bone geometry is correlated with oscillometric arterial stiffness in overweight older adults with low vitamin D" Rodriguez AJ, Xu CLH, McMillan LB, Srikanth V, Scott D, Ebeling PR. Poster #27 Winchester, England

- ENDO 2017 "Lower bone mineral density in the femoral neck is associated with greater abdominal aortic calcification and rate pressure product in healthy older Australians" Rodríguez AJ, Scott D, Hodge A, English D, Giles G, Ebeling PR. Oral abstract #29968. Orlando, USA

- ANZBMS-JSBMR-IFMRS 2017 "Effect of long-term leptin replacement therapy on bone mass in a pre-menopausal woman with genetic leptin deficiency" Rodríguez AJ, Paz-Filho GJ, Delibasi T, Ebeling PR, Wong M-L, Licinio J. Poster #199. Brisbane, Australia.

- WCO-IOF-ESCEO 2017 "Balloon kyphoplasty compared to percutaneous vertebroplasty: What is the evidence?" P. R. Ebeling, A. J. Rodríguez, H. A. Fink, L. Mirigian, N. Guañabens, R. Eastell, K. Akesson, D. Bauer Oral abstract #2264. Florence, Italy

- WCO-IOF-ESCEO 2016 "A systematic review and meta-analysis of randomised controlled trials on the effect of vitamin D supplementation on measures of arterial stiffness" Rodríguez AJ, Scott D, Srikanth V, Ebeling PR. Poster #236. Malaga, Spain

- ASBMR 2016 "Pain, safety and quality of life outcome of kyphoplasty for vertebral compression fractures" Rodríguez AJ, Fink HA, Mirigian L, Guañabens N, Eastell R, Akesson K, Bauer D, & Ebeling PR. Poster #LA-SA0378. Atlanta, USA

- ESA/SRB/ANZBMS 2016 "Relationship between low lean tissue and arterial stiffness: a systematic review and meta-analysis of observational data" Rodríguez AJ, Scott D, MD Nazmul Karim, Srikanth V, Ebeling PR. Oral abstract #36926. Gold Coast, Australia

- ANZBMS 2015 "Low relative muscle mass is associated with increased presence and severity of aortic calcification" Rodríguez AJ, Scott D, Khan B, Khan N, Hodge A, English D, Giles G, Ebeling PR. Poster #P2. Hobart, Australia

- AASD 2015 "Association between circulating adipocytokine concentrations and microvascular complications in patients with type 2 diabetes mellitus: a systematic review and meta-analysis of controlled cross-sectional studies" Rodríguez AJ, Paz-Filho G, Mastronardi CA, Neeman T, Nunes V. Oral abstract #Ab0045. Hong Kong SAR, China

- ESA 2015 "Circulating adipocytokine concentrations are associated with microvascular complications in patients with type 2 diabetes mellitus: a systematic review and meta-analysis" Rodríguez AJ, Paz-Filho G, Mastronardi CA, Neeman T, Nunes V. Oral abstract #197. Adelaide, Australia.

- ENDO 2015 "Association between leptin and diabetic microvascular complications: a meta-analysis of cross-sectional data" Paz-Filho G, Mastronardi CA, Neeman T, Nunes V, Rodríguez AJ. Poster THR-610. San Diego, USA

List of Additional Publications During Candidature

- Rodríguez AJ, Ebeling P "At what price increased mortality risk?" Osteoporos Int. (2018) Aug; 29(8): 1927-1928 doi.org/10.1007/s00198-018-4550-5

- Aleksova J, Rodriguez AJ, McLachlan R, Kerr P, Milat F, Ebeling PR. "Gonadal Hormones in the Pathogenesis and Treatment of Bone Health in Patients with Chronic Kidney Disease: A Systematic Review and Meta-Analysis" Curr Osteoporos Rep [Accepted]

- Rodriguez AJ*, Fink H*, Mirigian L, et al "Pain, quality of life and safety outcomes of kyphoplasty for vertebral compression fractures: report of a task force of the American Society for Bone and Mineral Research". J Bone Miner Res. (2017) Sep;32(9):1935-1944. doi: 10.1002/jbmr.3170 [Highest ranking journal in bone research]

- Rodríguez AJ "Vascular risk in familial Mediterranean fever". Anatol J Cardiol. (2017) Feb;17(2):139 doi: 10.14744/AnatolJCardiol.2016.22571

- Rodríguez AJ, Scott D, Ebeling PR "Comments on Li et al.: Meta-analysis of hypertension and osteoporotic fracture risk in women and men". Osteoporos Int. (2018) Jan;29(1):257-258. doi: 10.1007/s00198-017-4246-2

- Rodríguez AJ, Scott D, Ebeling P, Abrahamsen B "Reply to: systematic review and meta-analysis for the association of bone mineral density and osteoporosis/osteopenia with vascular calcification in women". Int J Rheum Dis. (2017) Dec;20(12):2144-2145 doi: 10.1111/1756-185X.12942

- Rodriguez AJ, Scott D, Ebeling P "Effect of weight loss induced by energy restriction on measures of arterial compliance: A systematic review and meta-analysis". Atherosclerosis 252:201–202. doi: (2016) Sep;252:201-202. 10.1016/j.atherosclerosis.2016.06.043

- Morton SK*, Rodríguez AJ*, Morris DR, et al "A Systematic Review and Meta-Analysis of Circulating Biomarkers Associated with Failure of Arteriovenous

Fistulae for Haemodialysis." PLoS One (2016) Jul 26;11(7):e0159963. doi: 10.1371/journal.pone.0159963

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* Asterisk indicates joint first author

List of Competitive Grants, Scholarships and Funding

2018   Barbara Mawer Travelling Fellowship, BRS (£1,900 ~ $3,370)

2018   Postgraduate Publication Award, Monash University ($4,600)

2017   Christine & T. Jack Martin Research Grant, ANZBMS ($12,900)

2015   Australian Government Research Training Stipend (formally - Australian Postgraduate Award) ($26,000 p.a. until 2018)

2015   Monash University, Department of Medicine, Faculty top-up Scholarship ($5,000 p.a. until 2018)

List of Honours and Awards

2018   ECTS Travel Grant (€300 ~ $480)

2018   ASBMR Young Investigator Award (US$1,000 ~ $1,700)

2018   Australian and New Zealand Bone and Mineral Society Travel Grant ($500)

2017   ENDO Outstanding Abstract Award ($250)

2017   ENDO Early Career Forum ($500)

2017   Australian and New Zealand Bone and Mineral Society Travel Grant ($300)

2016   ESA-IPSEN International Travel Award ($3,500)

2016   WCO-IOF- ESCEO-AgNovos Healthcare Young Investigator Award (€2,500 ~$4,000)

2016   Southern Clinical School (Monash University) 3-Minute Thesis – First Prize ($100)

2015 Asian Association for the Study of Diabetes International Travel Grant ($1,500)

2015 Australian and New Zealand Bone and Mineral Society Travel Grant ($250)

2015 Endocrine Society of Australia Travel Grant ($310)

Training and Skills Development

PhD Coursework

MPH5041 Introduction to Biostatistics

This unit introduces students to biostatistics as applied to public health and management studies. Biostatistics is the science of describing, summarising, and analysing health-related data. It is essential to understand biostatistics in order to design, conduct, and interpret health-related research. The basic principles and methods used in biostatistics are covered in this unit. This includes the technical qualifications necessary for analysing and interpreting data on a descriptive and bivariate level.

Topics included classifying health data; summarising data using simple statistical methods and graphical presentation; sampling distributions; quantifying uncertainty in results from a sample; working with statistical distributions; comparing two or more groups/methods using confidence intervals and hypothesis tests (p - values); assessing the association between an outcome and an exposure using the chi-squared test; using risk comparisons (RR and OR); predicting an event or identifying risk factors for an event of interest where the event is measured on a continuous scale or a binary scale (yes/no).

TRM6002 Translational Research

Translational research is a growing and exciting new discipline in medicine that deals with the development of fundamental scientific findings into tangible clinical

outcomes. Translational researchers are involved in identifying a worthwhile scientific finding that can be applied to a clinical setting. Along this research and development pipeline are a series of critical check-points that provide the investigator vital tools to generate a valuable result that has merit for translation. This unit will establish a fundamental knowledge in the processes involved in developing a basic science finding through to clinical studies. The unit provides workshop-based learning in the development of discipline-specific laboratory research questions and how they are applied to broader clinical applications. The main focus of this unit is to identify how fundamental scientific questions may have multidisciplinary clinical answers. Other core learning outcomes are through understanding how scientific concepts can be marketed and communicated effectively through research pipeline procedures and the responsibilities of the researcher that may be derived from this.

Stata

Stata is a powerful statistical analysis software that requires knowledge of statistical theory as well as simple computer coding. I self-learnt many *Stata* commands to analyse data including, Cox-proportional hazard models (survival analysis), meta-analysis and meta-regression including investigation of publication bias and heterogeneity, mediation analysis and advanced graphing techniques. Through using *Stata*, I have developed skills in database management and maintenance of patient records. In collaboration with a research group from Denmark, I also learnt a

technique called propensity score-matching to balance baseline characteristic between experimental and control participants in the observational setting.

Clinical Imaging

DXA – Dual-energy X-ray absorptiometry

Throughout my candidature I became proficient in bone density and body composition assessment by DXA. In-service training was delivered the Hologic Inc. Also, I secured a certificate from the Australia and New Zealand Bone and Mineral Society as proof of competency to operate the device, patient positioning and image acquisition as well as radiation safety. I am experienced in performing whole body composition scans, hip and spine bone density and lateral vertebral assessment for the purposes of quantifying aortic calcification.

TBS – Trabecular Bone Score

In addition to spine bone density acquisition, I undertook further training in acquiring TBS values for these images. TBS is a bone textural analysis. I secured a certificate of competency from Medimaps Group LLC.

pQCT- Peripheral quantitative computed tomography

I became proficient in volumetric bone density and muscle composition analysis through pQCT. Training for this was delivered by my supervisor Dr. David Scott who is an experienced operator. I learnt how to acquire images and run analysis scripts on the software to quantify bone, muscle and adipose tissue.

HR-pQCT – High Resolution peripheral quantitative computed tomography

I received training in HR-pQCT by ScancoMedical and completed a certificate of competency. HR-pQCT is a highly advanced bone microstructural analysis. I learnt patient positioning for radial and tibial scans, image evaluation and 3D rendering. Analysis including cortical and trabecular bone parameters.

Clinical trials

GCP – Good Clinical Practice

As part of the research programme of the Bone & Muscle Health Research Group a Phase 2 clinical trial of the investigational product bimagrumab sponsored by the pharmaceutical company Novartis AG. To be a trial site and an associate investigator I completed supervised training GCP and in the primary outcome measures of the study, including the Short Physical Performance Battery (SPPB) which is a suite of clinically relevant tasks that can be performed in an office such as walking tests and standing out of a chair.

Blood pressure monitoring

As part of the research programme of the Bone & Muscle Health Research Group an exercise trial of 50 obese/overweight older adults was currently active. As part of this study I had primary responsibility for cardiovascular measures in these participants. I learnt to operate an oscillometric blood pressure monitor (Mobil-o-Graph, IEM) capable of acquiring estimates of arterial stiffness.

Clinical trial co-ordination

As part of the research programme of the Bone & Muscle Health Research Group an exercise trial of 50 obese/overweight older adults was currently active. As part of this study I had primary responsibility for patient recruitment, managing and scheduling appointments, data management, liaising with a pharmaceutical company (Slade Compoundia) to supply medications (vitamin D or placebo), liaising with Monash Pathology for blood collection and analysis and being the primary point of contact for information on the study.

Human Research Ethics

I co-wrote an ethics submission (Approval number: HREC_15_MonH_182) for a trial of vitamin D combined with exercise for the improvement of exercise responsiveness in overweight and obese older adults with low vitamin D. I had responsibility for managing documentation and amending the protocol as needed.

Podcast

Together with my colleague from the Bone & Muscle Health Research Group, Dr Ayşe Zengin, I research, record, edit and manage a podcast entitled "Bone Group Banter". This podcast is publicly available and includes general discussion on musculoskeletal topics aimed at the general public. We interviewed esteemed researchers in the field and report from all conferences we attend with the latest research.

Statement of Aims

Overall aim:

This thesis aims to further understand the relationship between the musculoskeletal system and the vasculature through investigating:

- Associations of muscle and bone with vascular disease in older adults

- Effects of vitamin D supplementation and calcium intake on cardiovascular risk

Specific aims:

Chapter 2: Associations of muscle mass and function with vascular disease

This chapter investigates the association between muscle mass and function with arterial stiffness and aortic calcification. The specific aims of this chapter are:

- To review and synthesise the current evidence through meta-analysis regarding the association of muscle mass with measures of arterial stiffness in any population [Chapter 2.2]

- To investigate cross-sectional associations of muscle mass with AAC presence and severity in healthy older adults [Chapter 2.3]

- To investigate prospective associations of AAC with physical function decline in a cohort of postmenopausal women [Chapter 2.4]

Chapter 3: Associations of bone density with vascular disease and potential effects on the heart

This chapter investigates the association impact of aortic calcification on heart function and its association with bone density. The specific aims of this chapter are:

- To examine cross-sectional associations of bone density with cardiac workload in healthy older men and women and to determine if aortic calcification mediates this relationship [Chapter 3.2]

Chapter 4: Effects of micronutrient consumption on vascular disease and its underlying pathology

This chapter examines separately the effect of vitamin D supplementation in improving cardiovascular risk and the impact of calcium intake on cardiovascular mortality. The specific aims of this chapter are:

- To review and synthesise the current evidence through meta-analysis of the effect of vitamin D supplementation in randomised trials on:
  - Measures of arterial stiffness [Chapter 4.2] and;
  - Inflammation in patients with heart failure [Chapter 4.3]
- To investigate the impact of dietary calcium intake on all-cause and cardiovascular mortality in a prospective cohort study of healthy men and women [Chapter 4.4]

Chapter 1

Chapter 1.1 Introduction

Physical impairment during ageing is partly attributable to the loss of muscle mass and function. Muscular effects can also lead to skeletal effects including the loss of bone mass and increased risk of fracture. Older individuals in which sarcopenia and osteoporosis are common clinical features, are at risk of cardiac and vascular events and potentially hospitalisation and death.

As isolated systems, there is a wealth of understanding into how these characteristic features of ageing occur (namely cardiovascular disease, sarcopenia and osteoporosis) . This in turn has spurred the development of therapeutic strategies to target each of these features. However, as the population ages (particularly in developed nations) it has become increasingly important to address multiple pathologies as targeting isolated systems may lead to unwanted consequences in another system. Achieving a multi-system treatment necessitates addressing the shared nature of these pathologies.

To this end, we are increasingly understanding the shared nature of diseases that affect the skeleton, muscles and the vascular system. However we are still uncertain if bone and muscle diseases (which usually occur concomitantly) arise as a consequence of prevalent but subclinical cardiovascular disease or that cardiovascular disease contributes to, and potentially accelerates, the development

of musculoskeletal conditions. In short, are musculoskeletal disorders and cardiovascular diseases biologically linked or are their manifestations in older age just the inevitable confluence of age-related diseases? Little research has focussed on specific types of cardiovascular diseases and this review focusses on diseases which affect blood vessels, accounting for one-quarter of non-coronary deaths in Australia [25].

Overall, the following literature review is an exploration of (1) bone disease and its links to cardiovascular disease (2) muscle disease and its links to cardiovascular disease and (3) potential treatment strategies that target all systems.

Chapter 1.2: Narrative Review

Exploring the links between common diseases of ageing –osteoporosis, sarcopenia and vascular calcification

Rodríguez AJ, Scott D & Ebeling PR

Exploring the links between common diseases of ageing – osteoporosis, sarcopenia and vascular calcification

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Abstract

Vascular diseases account for a significant proportion of preventable deaths, particularly in developed countries. Our understanding of diseases that alter the structure and function of blood vessels such as vascular calcification and vascular stiffness has grown enormously such that we now appreciate them to be active processes that can be modified. Interest has also grown in examining the links between other diseases of ageing such as the loss of bone (osteoporosis) and muscle (sarcopenia) with the development and progression of vascular disease as these three disease states commonly co-occur in older age. Cardiovascular disease (including calcification and arterial stiffness) is highly prevalent in older populations and it appears that its progression is accelerated in patients with osteoporosis, fracture, sarcopenia and in those who are functionally impaired. Biological and clinical evidence supports a view that vascular disease (calcification/stiffness) may be both a cause and consequence of diseases of ageing including musculoskeletal decline. This review provides an overview of the development of vascular calcification and stiffness and explores the molecular and physiological mechanisms linking osteoporosis and sarcopenia to vascular disease development. This review also examines clinical evidence supporting the association of muscle and bone loss with vascular disease and concludes by reviewing the interventional and therapeutic potential of bone-active minerals and hormones (calcium and vitamin D) on cardiovascular disease biology, given these represent potential interventions to target multiple body systems. Overall, this review will aim to highlight the underappreciated burden of cardiovascular disease in individuals in the context of musculoskeletal diseases.

Part 1 Introduction: Osteoporosis, Sarcopenia and Vascular Disease: Shared Risk Factors and Clinical Associations

The loss of bone and muscle mass and the development of cardiovascular disease appear to be the inevitable consequences of ageing. Each of these body systems declines through defined mechanisms, yet we are increasingly understanding the shared nature of diseases that affect the skeleton, muscles and the vascular system. It is uncertain if bone and muscle diseases (which usually occur concomitantly) arise as a consequence of prevalent but subclinical cardiovascular disease or that cardiovascular disease contributes to, and potentially accelerates, the development of musculoskeletal conditions. In short, are musculoskeletal disorders and cardiovascular diseases biologically linked or are their manifestations in older age just the inevitable confluence of age-related diseases? The following review is an exploration of (1) bone disease and its links to cardiovascular disease (2) muscle disease and its links to cardiovascular disease and (3) potential treatment strategies that target all systems.

1. Osteoporosis
   a. Definition

Osteoporosis is a systemic skeletal disorder characterised by the loss of bone mass and deterioration in bone microarchitecture. These structural and molecular changes in bone biology combine to compromise bone strength leading to skeletal fragility and increased susceptibility of fracture [1]. Recovery from a fracture is costly, takes many months and indeed some never truly recover as they may lose independence and the risk of mortality greatly increases following a fracture [2]. Bone is a dynamic organ that is constantly remodelling in response to the stresses of living. During ageing, gradual bone loss occurs from the relative increase of bone resorbing osteoclast activity to new bone-forming osteoblast activity. Due to the increased osteoclast activity relative to osteoblast activity, there is a deficit in new bone formation and this deficit compounds over time leading to skeletal fragility [3]. Clinically, osteoporosis is diagnosed either from sustaining a minimal trauma fracture (that is a fracture occurring following a fall form a standing height or from a minimal force applied such as bumping into a desk) or if the T-score of bone mineral density (BMD) test from scans of the hip and lumbar spine by dual-energy x-ray absorptiometry (DXA) fall below -2.5 [Figure 1]. In the context of BMD testing, a T-score is a statistical measure of the relative distance of measured BMD from the BMD of a young, healthy person. T-scores between -2.5 and -1.0 are considered to be *osteopenic*, that is, evidence of bone loss but not severe enough to greatly increase fracture risk but closer monitoring is warranted. Scores above -1.0 are considered "normal" or "ideal".

Osteoporosis	Osteopenia	Normal bone density
-4.0 -2.5	-2.0 -1.0	+3.0
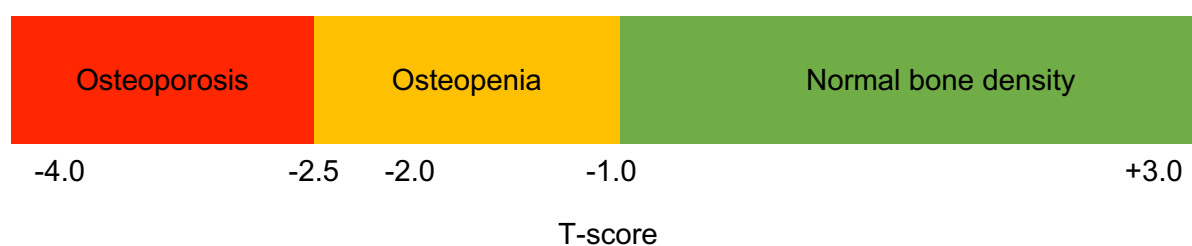
T-score

Figure 1. Classification of bone mineral density based on T-score.

In Australia, recent data suggest that approximately 2% of men and approximately 10% of women have osteoporosis based on hip BMD [4]. Considering the lumbar spine as well (hip or spine DXA derived BMD) these numbers increased to 6% in women and 22% in women. Historical data has shown that in Australia, the (age-adjusted) rate of hip fractures in women is 25 per 10,000 person-years and 9 in men; similarly, the rate of vertebral fractures is 19 per 10,000 person-years and 7 in men [5]. Overall, this represents a substantial disease burden and indeed in Australia it is estimated that hip fractures cost an average of $23,000, and potentially over $33,000 in health care expenditure [6]. These data are replicated globally, where large-scale analyses have shown that there are approximately 3.5 million new fragility fractures every year in the European Union costing €37 billion annually and there are similar trends in the United States [7,8].

b. <u>Management</u>

Management of osteoporosis usually begins with preventative strategies such as addressing modifiable lifestyle elements through promoting more physical activity and adequate intakes of calcium and vitamin D. Calcium and vitamin D have been shown to have only modest benefits in terms of increasing bone mass and reducing fracture rates[9]. They are nonetheless the standard, first line recommendation for individuals with concerns over their bone health largely due to their low cost and proven safety, though calcium alone (either through supplements or through dietary means) has raised concerns about the potential for increased cardiovascular events [10,11]. Pharmacological interventions to treat bone loss broadly fall into two categories: anti-resorptives and anabolics. Anti-resorptive medications seek to shift the bone resorption-formation balance toward more formation by inhibiting elements of bone resorption. There are two main types of anti-resorptives: bisphosphonates and denosumab. Bisphosphonates are a class of compounds so-named as the basic structure of the chemical has two phosphate groups and two substituents and combinations of which distinguishes the bisphosphonate type [12]. Bisphosphonates produce their anti-resorptive effects by binding, through the two phosphate groups, with high affinity to bone matrix which is rich in calcium. Bisphosphonates are structurally similar to pyrophosphate, the mineralised constituent of bone matrix. Bisphosphonates are taken up by osteoclasts as part of the resorption process and disrupt the enzyme farnesyl diphosphate synthase in the HMG-CoA reductase pathway (also known as the mevalonate pathway) in the osteoclast causing cell apoptosis from an inability to transcribe proteins necessary for cell membrane formation[13]. Denosumab is a human antibody to the receptor activator of nuclear factor kappa-β ligand (or RANKL). RANKL binds to receptor activator of nuclear factor kappa-β (RANK) on the surface progenitor cells of osteoclasts (pre-osteoclasts) promoting their differentiation into mature osteoclasts. Inhibition of the process, restricts osteoclast maturation and ultimately reduces bone resorption [14]. Thus, denosumab mimics the action of osteoprotegerin (OPG), a natural inhibitor of RANK-RANKL interactions. The other category of osteoporosis medications are anabolic therapies which seek to promote bone formation. Teriparatide is a recombinant protein of human parathyroid hormone (PTH) consisting of the first 34 amino acids of the N-terminus (the active part of the compound). Teriparatide works by transiently stimulating osteoblast activity by increasing serum calcium concentration [15,16]. Chronically elevated calcium levels

(typically seen in cases of hyperparathyroidism) result in reduced BMD, thus teriparatide is administered intermittently to stimulate the inhibition of osteoblast apoptosis. Strontium ranelate is another anabolic agent and is unique in that it has the dual-action of also inhibiting bone resorption [17]. Strontium ranelate's mechanism of action is to stimulate calcium sensing receptors leading to the maturation of pre-osteoblasts to osteoblasts capable of bone formation. It also stimulates osteoblasts to secrete OPG which inhibits osteoclast maturation as outlined above.

c. <u>Risk factors for osteoporosis and relationship with cardiovascular disease and mortality</u>

The risk factors for osteoporosis include, a family history of fracture, advanced age, history of falls, smoking, inadequate calcium and vitamin D intake, low amounts of physical activity and alcohol consumption [18]. These risk factors and their contribution to fracture risk have been incorporated into risk assessment tools such as FRAX® and the Garvan Fracture Risk Calculator [19,20]. Interestingly, obesity (commonly co-exiting with these risk factors) is not a classic risk factor for osteoporosis and indeed seems "protective" against low bone density. The traditional explanation for this is that as bone is a dynamic organ, its cells are responsive to loading and other stresses from the environment [21]. In the context of obesity, the increased weight of the individual will create increased loading on the bones which over time translates into a higher bone density. Bone density is not the only feature of bone strength. Indeed, the bone microarchitecture of obese individuals is compromised and this has been demonstrated clinically and pre-clinically [22,23]. Importantly, the majority of fractures in the population actually occur in overweight or obese individuals [24]. Obesity increases the risk of falls, mobility limitation and functional impairment, which may contribute to the risk for loss of bone density and increased fractures. Often, this is attributable to the phenomenon whereby as we age, muscle tissue is steadily lost and fat tends to accumulate (within and between the muscle fibres known as inter-intramuscular adipose tissue) such that relatively, the proportion of fat to muscle in the limbs increases with age [25]. Therefore, the remaining muscle mass may be insufficient to move the obese individual's frame. Previous literature has demonstrated that in community-dwelling older adults, increasing visceral fat percentage and total body fat percentage was negatively correlated with muscle density (a proxy measure of inter-intramuscular adipose tissue) [26]. That is to say, the more adipose tissue in the body, the more appears in the muscle (lower muscle density is indicative of higher amounts of fat accumulation). Additionally, in another sample of community-dwelling older adults, calf-muscle density positively correlated with cortical volumetric BMD in the proximal tibia (an area rich in cortical bone) in men and was positively associated with cortical volumetric BMD and cortical area in the proximal tibia in obese women after multivariable adjustment [27]. That is to say, with increasing muscle density (hence less fat infiltration), there is greater amounts of cortical bone mass. It is therefore important to also consider obesity as a risk factor for poor bone health. If obesity is incorporated into the milieu of factors predisposing to osteoporosis and fracture this consequently means that the majority of risk factors predisposing to cardiovascular, and in particular vascular, diseases, are also risk factors for osteoporosis [Figure 2]. It is unsurprising then that much epidemiological evidence supports a shared bone-vascular disease axis. Numerous observational studies have shown that low bone density can predict cardiovascular disease and events [28,29]. Equally, the risk of falls and fractures is increased in

individuals with high blood pressure and elevated central stiffness [30]. Given this seemingly bi-directional relationship, it is difficult to determine what is the cause and what is the effect. Taking a life-course approach to understanding the development of osteoporosis and vascular disease would point to auxiliary factors that influence both bone heath and vascular health potentially earlier in life that may help explain their co-existence in older age. During ageing, and particularly in the growth phase of life, muscle mass and function is a crucial determinant of bone mass and strength and is also associated with a more favourable cardiovascular health profile [31]. Thus, understanding the relationship between the musculature and the vasculature may help unlock critical new pathways to further our knowledge of the bone-vascular axis.
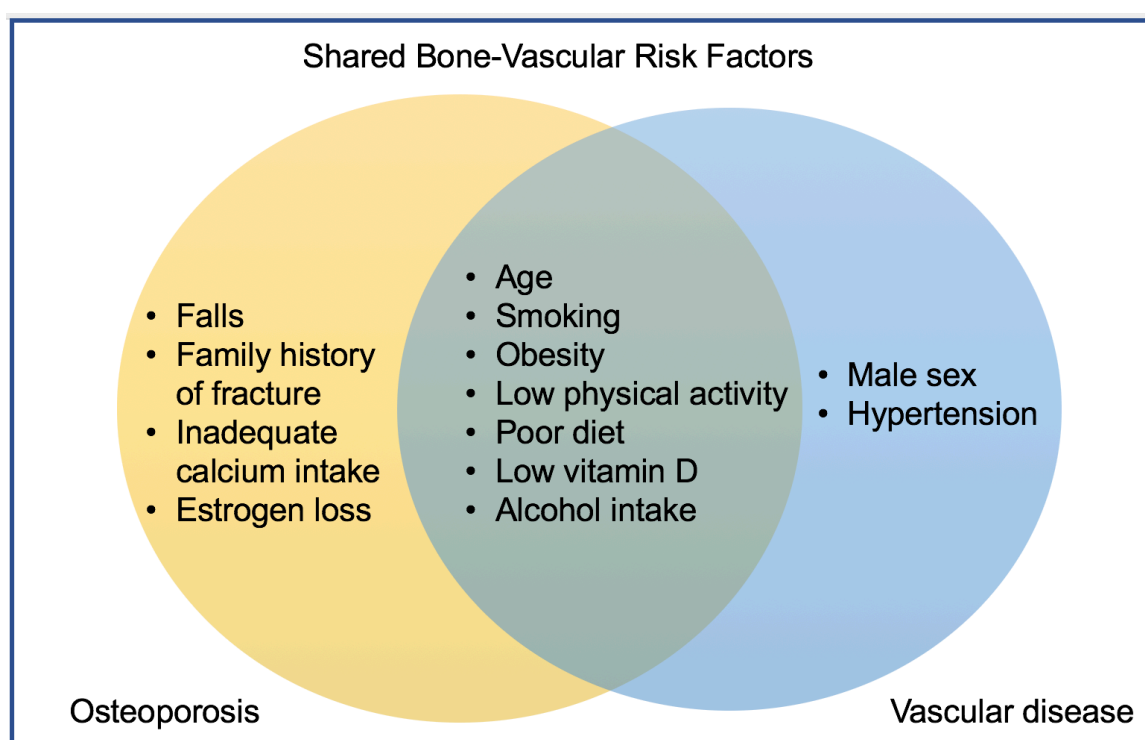
Figure 2. Shared and unique risk factors of osteoporosis and vascular disease

Table 1. Clinical evidence supporting bi-directional relationship between bone loss and vascular disease

Direction of relationship	Summary of findings	Reference
Bone → Vascular	Low bone density cross-sectionally associated with higher blood pressure	32
	Low bone density predicted development of echogenic carotid plaques	33
	Diagnosis of osteoporosis/low BMD predicts vascular events	34, 28

	Vascular events were more common in those identified with a history of fracture	29
Vascular → Bone	Diagnosis of atrial fibrillation increases risk of hip fracture	35
	High blood pressure leads to greater bone loss	36
	Fractures more likely in those with high blood pressure	30,37
	Vascular calcification predicts bone loss and fracture risk	38
	Carotid plaques predicted non-vertebral fractures	39

2. Sarcopenia

    a. Definition

Sarcopenia describes the progressive loss of muscle mass and function leading to eventual frailty and increased risk of falls and fracture [40]. Previously, it was thought that the onset of physical frailty was an inevitable consequence of ageing. As life expectancy increases, particularly in developed countries, increasing attention has been paid to increasing the quality of life in older age as we are now living a substantial portion of our lives in this age group. One of the areas to address this need is in maintaining good physical function in older age. Foundational to this strategy is to preserve muscle mass and quality during ageing, where like bone, the intention is to capitalise on the growth phase of life to achieve the highest peak muscle mass achievable. As such, our understanding of the impact of age-related decline in muscle mass and quality has greatly increased. There are currently a number of "consensus" definitions for sarcopenia but despite this heterogeneity, sarcopenia is recognised as a robust predictor of falls and mobility limitation [41]. The European Working Group on Sarcopenia in Old People (EWGSOP) has gained preference in the field and using this definition, prevalence of sarcopenia in older adults has ranged from 5% to 22%, where age and ethnicity significantly impact on disease prevalence [42,43]. Recently, sarcopenia was given an ICD-10 code (M62.84) which many believe will facilitate recognition by caregivers and the uptake of intervention strategies. The development of sarcopenia is complex, but it is understood to involve a range of factors including sedentary lifestyle and micronutrient deficiencies such as vitamin D, amino acids and also macronutrients such as proteins which are required to maintain muscle tone and growth [44]. At the molecular level, the gradual loss in the regenerative capacity of muscle tissue is one of the better described pathways of muscle loss. Increased oxidative stress and chronic inflammation contribute to the differentiation of regenerative satellite cells into non-contractile, non-functional adipose tissue [45]. Recent data has suggested that this intra-/intermuscular adipose tissue has utility in predicting non-skeletal outcomes such as cardiovascular disease and is independently associated with inflammation [46,47].

b. Management

Muscle, like bone, is a dynamic organ and responds to use and disuse. As such, the most effective strategy to limit the loss of muscle mass and strength is through exercise training. Exercise has proven effectiveness across all ages throughout the life-course including in the very old and frail. Whilst bone responds most favourably to loading or impact type activities, most forms of physical activity (aerobic, resistance or weight training, incidental activities) appear to have positive effects on the musculature [48]. In older adults, walking is predominant as the most common source of physical activity. It has been observed that in individuals reporting the highest amounts of physical activity also have greater amounts of lean mass and muscular strength[49]. Consequently, interventional studies involving aerobic, resistance and multimodal (combination of different forms exercise into the one regime) have proven benefits in improving muscle mass and function, even in the very old at high fracture risk [50,51].

Nutrient consumption is also an important contributor to muscle mass and strength. Whilst total caloric consumption does not necessarily decline with advanced age, it is the composition of that consumption that contributes to muscle loss [52]. Importantly protein represents an increasingly smaller proportion of daily dietary intakes as one ages . Protein provides the necessary amino acids required for building new muscle and it has been shown cross-sectionally that low protein consumption is associated with lower muscle mass in older age [53,54]. Additionally, calcium and vitamin D are critical for muscle function. Calcium is required for neuromuscular transmission and vitamin D has a multitude of functions including, primarily, supporting calcium uptake and also has roles in muscle tone [55,56]. Calcium and vitamin D intake also appear to decline with advancing age further contributing to musculoskeletal decline and indeed sarcopenia is highly prevalent in individuals with low intakes of these micronutrients [57]. Interventional studies have demonstrated that protein supplementation may be able to support muscle mass increases in the elderly and that calcium and/or vitamin D supplementation may also have a positive effect on sarcopenia[58,59]. Indeed combining these nutrients, for example, protein supplementation combined with vitamin D supplementation, has resulted in significant gains in skeletal muscle mass, muscle strength and some markers of inflammation in sarcopenic older adults [60,61]. There is also some evidence to suggest that that combining nutrient supplementation with an exercise component can result in beneficial effects on sarcopenia [62,63].

c. Relationship between sarcopenia and cardiovascular disease and mortality

As muscle and bone share an intimate relationship, many of the risk factors linking bone and vascular diseases are similarly shared between sarcopenia and vascular disease [64]. Furthermore, similar to the relationship between osteoporosis and cardiovascular disease, much epidemiological evidence has demonstrated that sarcopenia and the components of sarcopenia individually are associated with cardiovascular disease and mortality. For example, in a cohort of 4,252 community-dwelling older men sarcopenia was associated with increased risk of mortality [hazard ratio (HR): 1.41; 95% confidence interval (CI) = 1.22-1.63] and those who were obese also had increased risk of mortality [1.21; 1.03-1.42]. Interestingly, there appeared to be a synergistic effect of the coincidence of sarcopenia and

obesity on mortality with an approximate 72% increased risk of all-cause mortality [1.35-2.18] [65]. Other longitudinal studies have demonstrated that in older adults [n=1512], the risk of cardiovascular mortality was greatest in those with low skeletal muscle mass [2.16: 1.51-3.08] [66]. These data indicate that muscle (as well as bone explored above) have independent effects on the development of cardiovascular diseases which would point to shared mechanisms (mechanisms common to both muscle and bone health and disease may be important for cardiovascular disease). Furthermore, nominal strategies that address osteoporosis and sarcopenia such as improving bone and muscle mass through exercise and micronutrient supplementation also have well established profound effects on the cardiovascular system. This then leads one to speculate that we may be observing related processes: that there exists pathobiological links between the development of cardiovascular diseases, osteoporosis and sarcopenia. By understanding the commonalities between the skeletal, muscular and vascular systems, we may be able to identify common pathways which lead to diseases in all three systems and thus be able to develop a strategy to target these pathways and thus all three systems. In designing these strategies, it is important also to look at the nature of cardiovascular disease in older age as, generally, a large proportion of these cardiovascular disease effects the function and structure of blood vessels, are usually the result of chronic lifetime exposure to risk factors that compound to eventually manifest in older age.

Part 2: Diseases of Blood Vessels

    1.   Underline: What is vessel disease?

          1.2 Definition

The vasculature is a complex network of conduit vessels. The structure (including size, shape and cellular architecture) of each vessel ultimately governs its purpose and function. Thus, diseases that affect the structure and function of blood vessels are inherently detrimental to human health. Indeed, vessel disease is the third largest cause of non-coronary cardiovascular deaths in Australia totalling 4,085 of the 17,426 (~23%) of non-coronary deaths in Australia in 2018 [67,68]. Vascular disease can be broadly defined as diseases that alter the micro- and macro-anatomical composition of the vessel affecting its usual function and performance. Principally the ageing aorta suffers two main fates: the vessel can become calcified and stiffen over time or the vessel walls can become weakened, expand (aneurysm) and eventually rupture. Indeed, the aorta is one of the first sites in which these structural and functional changes occur. Understanding of the fundamental biology and anatomy of the aorta is important if one is to understand the potential links of vascular disease to musculoskeletal diseases in ageing.

    2.   Anatomy and biology of the aorta

The aorta serves two important cardio-physiological functions: one as a major conduit for blood flow and another as a buffer against large transient increases in pressure created during systole, also known as the Windkessel effect. The Windkessel function of the aorta ensures smoothing of arterial pressure throughout the cardiac cycle. The aorta itself is muscular, has a normal diameter of around 30mm and

has its own blood supply. The structure of the aortic wall comprises three distinct layers [Figure 3] [69]. The innermost layer (*tunica intima*) is a single cell thick lining of endothelial cells that are highly responsive to stimuli including circulating factors and also the sheer stresses from blood flow. The middle layer (*tunica media*) is the thickest layer of the aortic wall. The predominant cell type in this layer are the vascular smooth muscle cells (VSMC). These are a highly specialised cell type that are capable of contraction as necessary during systole and have the ability to trans-differentiate into a proliferative and osteochondrogenic phenotype [70–72]. Interlaced between the VSMC are fibres of elastin and collagen. Elastin lamellae are concentrically arranged and are visible under light microscope, offering the aorta elastic abilities, stretching and relaxing according to tension and compression stresses throughout the cardiac cycle. Collagen fibres are arranged transversely along the length of the aorta providing structural integrity to the vessel and experience high tensional stress during diastole to promote blood flow. The outermost layer (*tunica adventitia* or *externa*) is mainly comprised of collagen supported by some elastin fibres. Its main function is to anchor the vessel to surrounding structures. Importantly, the elastin-collagen ratio changes along the length of the aorta[73]. There is a higher elastin content in more proximal regions of the aorta and the content of collagen progressively increases in the more distal and descending regions of the aorta. This is significant because given the differences in elastic potential across the aorta, some regions are more susceptible to vascular damage than others[74].
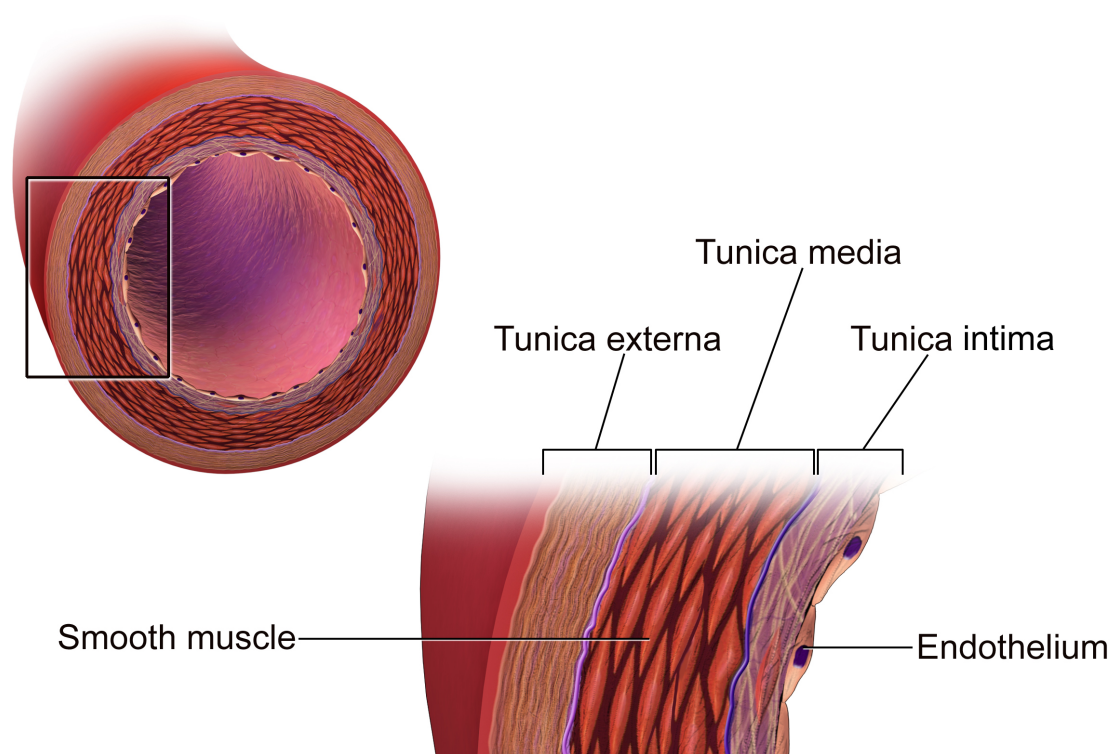
Figure 3. Layered structure of an artery.

3. Fate of the ageing aorta: Vascular calcification and stiffness

Of the fates of the ageing aorta, calcification is more a common disease than aneurysm; it is prevalent in up to 60% of healthy older individuals whereas the prevalence of aortic aneurysm is approximately 5% of older adults [75,76]. Vascular calcification is a macro-anatomical disease of blood vessels that has a long history in humans. In the Tyrolean Ice Mummy discovered in northern Italy, an autopsy of the remains detected substantial calcium deposits in the abdominal aorta [77]. The remains are thought to be close to 5,300 years old. Vascular calcification is considered to be a multifaceted disease, but in general is defined by the layer in which the calcification appears. Intimal calcification, occurring in the intimal layer of the aortic wall is associated with atherosclerosis which is characterised by lipid-laden plaques that have become deposited in the aortic wall and surrounded by a highly inflammatory environment including leukocytes and other immune cells; predominantly macrophages[78]. This type of calcification represents the prototypical lesion to which the majority of historical investigation of calcified arteries has been dedicated. The calcification is phenotypically eccentric and occludes the lumen. Medial calcification, sometimes also referred to as Mönckeberg's syndrome or medial calcific sclerosis occurs in the medial layer of the aorta and this type of calcification is driven largely by VSMC deposition of mineral in the wall [79]. The calcification present is usually concentric and associated with inflammation in the adventitia. Site is significant when it comes to vascular calcification. Calcification of large, central arteries and smaller, peripheral arteries is associated with different disease states but generally calcification in larger vessels is indicative of calcification in smaller vessels [80]. Calcification in the abdominal aorta is one of the first sites in which calcification develops and thus may act as a sentinel site for more generalised vascular disease[81]. As such, a majority of the discussion in this review will be dedicated to the understanding of abdominal aortic calcification (AAC).

The abdominal aorta is unique in its biology. As alluded above, the higher collagen to elastin content in this region of the vessel renders it susceptible to vascular injury. Localised disruption in the aortic wall from collagen and/or elastin degradation which is mediated by resident matrix metalloproteinases 2 and 9, can weaken the vessel wall and promote dilatation and aneurysm development[82]. Degradation of aortic wall products promotes macrophage (and other leukocytes) homing at this site to clear potentially immune-reactive particles [83]. Ineffective clearance of these products (apoptotic bodies) appear to be a preferential site for nucleation of calcium-phosphate crystals by pro-inflammatory macrophages. More generalised damage such as the cross-linking of collagen fibres which seems to occur with chronic hyperglycemia or as part of a chronic immune process can stiffen the aorta (arteriosclerosis) compromising the aorta's Windkessel function. Bioengineering studies have demonstrated that increased collagen content and the presence and composition of fibrotic/atherosclerotic lesions alters (increases) the strain modulus of blood vessels[84]. It naturally follows that calcification in and on the aortic wall, would result in a loss of compliance (stiffening), resulting in increased pressure retention throughout the vasculature manifesting as elevated systemic blood pressure (hypertension). Ultimately, this may raise blood pressure and contribute to ventricular hypertrophy which has profound implications

for cardiac risk [85,86]. Therefore, calcification in the aorta may be seen as a _cause_ of adverse haemodynamics resulting in elevated cardiovascular risk.

Stiffening may also precede calcification. Stiffness may directly impact on blood flow characteristics and affect target organs and tissues which, over time, result in the deposition and accumulation of ectopic minerals to fortify damaged vessels leading to calcification [87]. This theory would suggest that aortic calcification may in fact be a _consequence_ of adverse haemodynamics. Increases in systemic blood pressure may expose endothelial cells, which are highly mechanosensitive, to excess or abnormal wall sheer stresses leading to their dysfunction (characterised by increased cell surface expression of integrins and other chemotactic markers) [88,89]. Sustained excess sheer stresses on the endothelium may result in vascular injury which is the key trigger for promoting an immune-inflammatory response. Part of this response is to repair and remodel the aortic wall which may involve degradation of the elastin and collagen fibres exposing previously intracellular/intramural substances to the circulating immune system. The result of this is further inflammation and cellular infiltration and ultimately plaque formation. Macrophages are a key cellular infiltrate and as aforementioned, ineffective clearance of apoptotic bodies can trigger calcium-phosphate crystal formation. Generally, calcification of plaques is viewed as an attempt by the body to protect or stabilise the plaque and 'hide' potentially immunoreactive substances under a robust layer of fibrotic/calcific cap [90]. Given this pathobiological background there appears a number of commonalities as well as differences between these two related vascular features, calcification and stiffness, and these are summarised below [Table 2].

Table 2. Commonalities and differences between vascular calcification and stiffness

| Domain | Associated factor | Vascular feature | |
		Calcification	Stiffness
Clinical risk factors and features	Age	+	+
	Male	-	+
	Smoking	+	+
	Diabetes	+	++
	Hypertension	+	?
	BMI	?	++
	CKD	++	+
	Osteoporosis	+	?
	Sarcopenia	?	?
	Inflammatory diseases	?	+
Responsive to intervention	Anti-hypertensive	Likely yes	Yes
	Anti-inflammatory	Unknown	Unknown
	Physical activity	Unknown, likely yes	Yes
	Bone active medications	Inconclusive evidence	Unknown
	Calcium	Likely no	Unknown
	Vitamin D	Unknown	Inconsistent evidence
Pathobiological features	Vascular tissue affected	Intimal and medial layers	Intimal (uncertain)
	Major cell type involvement	VSMC, macrophages, myofibroblasts (uncertain), cOP	Endothelial cells, cOP, VSMC

Circulating & tissue factors	Increased	BMP-2, matrix Gla protein, oxLDL	AGEs, Elastin degradation products, MMP-2/-9
	Decreased	Wnt inhibitors, feutin-A	NOx, vitamin D secretion by endothelial cells
Bone mineral metabolism (concentration)	Calcium (tissue)	Elevated	Unknown
	Calcium (circulating)	Unknown	Unknown
	Vitamin D	Lowered	Lowered
	Phosphate	Elevated	Elevated (uncertain)
	PTH	Elevated	Elevated (uncertain)
	Estrogen/Estradiol	Lowered	Lowered (uncertain)

+ = increases risk; - = decreases risk; VSMC = vascular smooth muscle cell; cOP = circulating osteoprogenitor cells; BMP-1 = bone morphogenic protein-2; matrix Gla = matrix gamma-carboxyglutamic domain; oxLDL; oxidised low-density lipoprotein; Wnt = Wingless/Integrated; AGEs = advanced glycation endproducts; MMP = matrix metalloproteinase; NOx = nitric oxide; PTH = parathyroid hormone; BMI = body mass index; CKD = chronic kidney disease

4. <u>Imaging and quantifying calcification and arterial stiffness</u>

Investigation of aortic disease is part of routine clinical practice for those at high vascular risk. Imaging of the aorta can be achieved through a number of modalities including magnetic resonance imaging (MRI), quantitative computed tomography (qCT), lateral spine radiography and lateral spine densitometry. Functional assessment of the aorta can be achieved by functional MRI (fMRI), fludeoxyglucose F18 (^{18}F-FDG) positron emission tomography (PET) or more commonly, Doppler ultrasound. Doppler ultrasound imaging is inexpensive and can be utilised in a variety of vascular regions including the abdominal aorta, it has the advantage of being able to view vessel with and without evidence of plaque. Recently however, densitometry is gaining popularity as the imaging modality of choice due to the low radiation exposure and rapid image acquisition and the opportunity to screening for cardiovascular disease at the time of bone density and vertebral fracture assessment [91]. Next-generation densitometric devices have a lateral enable function which allows image acquisition with a patient lying in a supine position facilitating image acquisition in older individuals and individuals who may have difficulty in laying in a foetal position. Once detected, a semi-quantitative scoring system is used quantify the extent of calcification. The most commonly used scoring system is the *AAC24* where scores can range from 0-24 [92]. This system involves examining the extent of calcific deposits on the anterior and posterior aspects of the aortic wall in the L1-L4 vertebrae [Figure 4]. In this technique, for each vertebral segment, calcified deposits are scored 0 for no evidence of calcification; 1 — if one-third or less of the wall is calcified; 2 — between one-third and two-thirds of the walls are calcified and 3 — more than two-thirds of the walls are calcified. The scores for the anterior and posterior walls are summed meaning each vertebral segment contributes a maximum of score of six to the final score [92]. The final aortic calcification score (ACS) is a composite of all four vertebral segments ranging from a minimum "0" to a maximum of "24". Scores are non-normally distributed and thus ACS is considered a non-continuous outcome. It is important to consider the severity of calcification. Based on the ACS, severity of calcification is defined according to three groupings: "no calcification" (ACS = 0), "moderate calcification" (ACS between 1 and 5), and "severe calcification" (ACS ≥6).
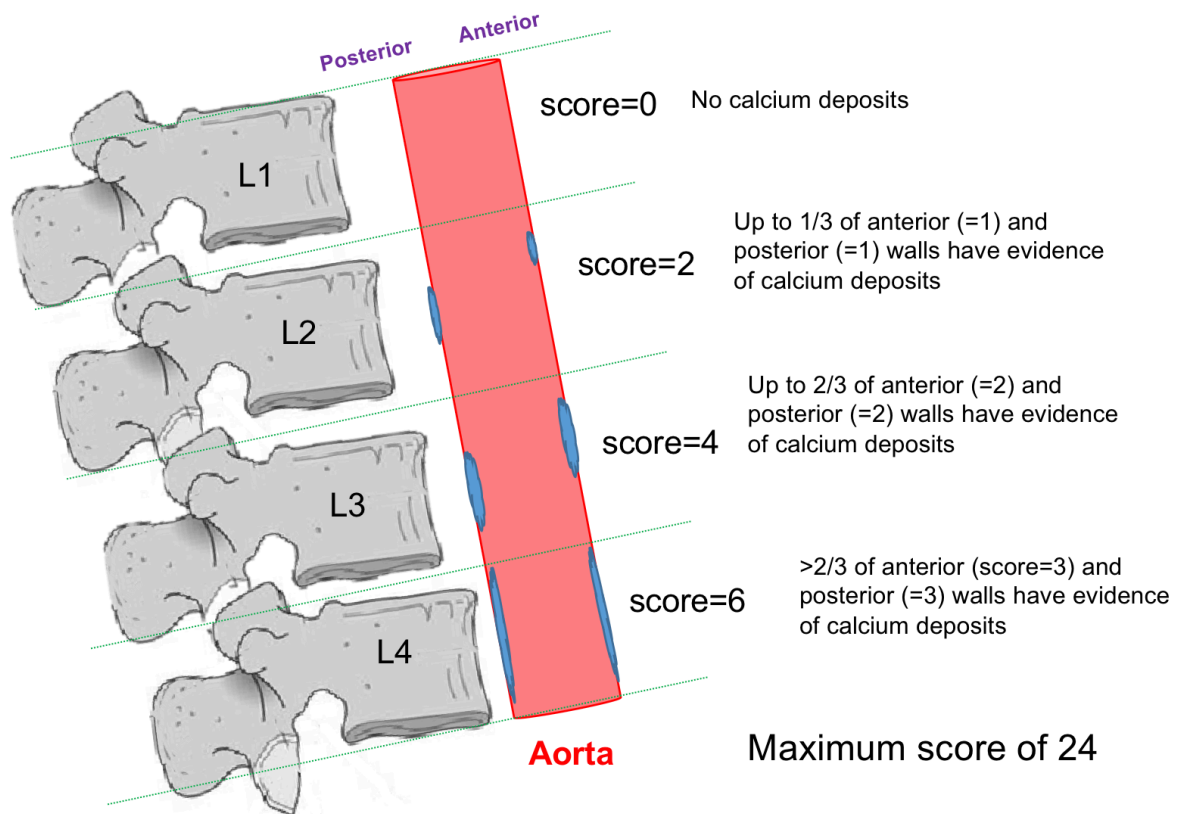
Figure 4. AAC24 scoring system

Vascular calcification adversely affects the elastic properties of blood vessels and though the stiffening of vessels cannot be seen, it can be quantified. There are a number of parameters which serve as useful estimates of vessel stiffness. Pulse pressure (PP) is the difference between the systolic (pressure at ventricular contraction) and the diastolic (pressure between contractions) blood pressure. A narrow PP usually reflects central vascular stiffness most likely related to calcification of the aorta. This can be measured from a standard office blood pressure monitor (sphygmomanometer). Pulse wave velocity (PWV) is the composite of the forward pressure wave created by ventricular contraction and a reflected wave from a distal site [Figure 5] [93]. The gold standard measurement of arterial stiffness is the carotid–femoral PWV determined by tonometry or by intra-aorta catheter. This is estimated using the foot-to-foot velocity method whereby transcutaneously, the right common carotid artery and the right femoral artery and the time delay (or transit time, Dt) are measured (in seconds, s) between the feet of the two waveforms. A variety of different waveforms can be used including Doppler, pressure and distension. The distance (L) covered by the waves between these two sites is measured (in metres, m), and PWV is then calculated as ¼ L/Dt with the unit m/s. The less compliant the arteries, the faster the reflected wave returns augmenting systolic pressure interpreted as an increased PWV. The extent of this augmentation in systolic pressure is called the augmentation index (AI). These measures of arterial stiffness can also be quantified using oscillometric devices which have shown strong agreement with classic techniques [94].
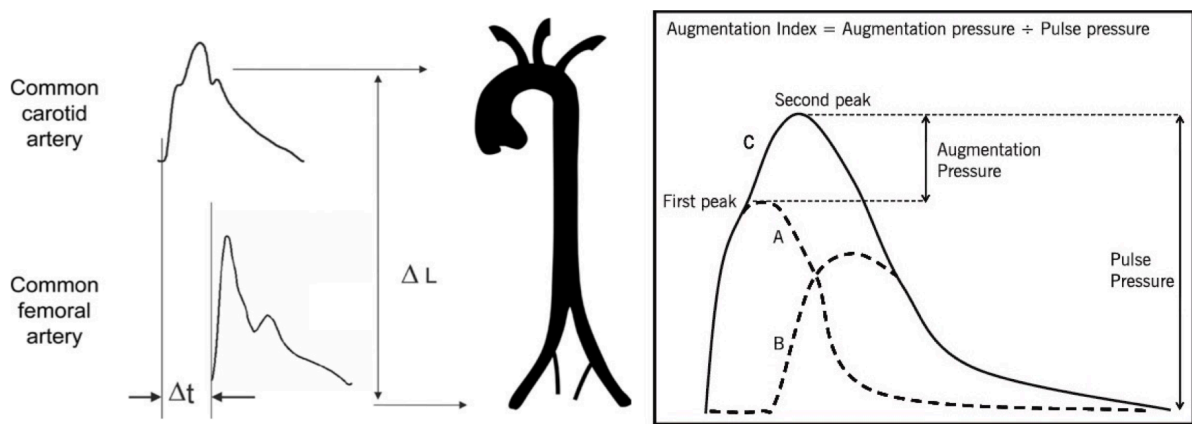
Figure 5. Arterial Stiffness: Example wave forms explaining derivation of PWV (left) and AI (right) – adapted from Laurent et al. 2006 [95].

5. Cardiovascular risk related to vascular calcification and stiffness

Vascular calcification and stiffness are well-established markers of cardiovascular disease and can both independently predict future cardiovascular events and mortality [93,96,97]. Whilst this finding has been demonstrated in multiple populations, our understanding of how these vascular features impose an increased risk is not precisely understood. It is generally accepted that calcification and stiffness place an extra burden on the heart as it must work harder to overcome peripheral resistance. Overtime, remodelling of the heart to cope with chronic over-burden increases the risk of adverse cardiac events. Several studies exist which support this understanding [Table 3]. A study in older men with high cardiac risk but preserved ejection fraction, calcification in both the thoracic and abdominal aorta was associated with a number of echocardiographic parameters and the total aortic calcification was positively associated higher left ventricular mass [85]. This finding supports the view that calcification in the large vessels, particularly more central vessels, create extra peripheral resistance for which the heart has to work against in order to maintain adequate supply and tissue perfusion. Though, other studies investigating calcification in coronary arteries have not shown this suggesting that calcification may have a site-specific effect on left ventricular mass [98]. This may be explained by the aorta receiving a large volume of blood and pressure direct from the heart during each cardiac cycle and as such stiffness in the aorta would have a more pronounced effect on overall cardiac work. It would be informative to understand if other conditions of ageing associated with increased cardiovascular risk and mortality (particular those that appear to co-exist with vascular calcification and stiffness) if they share similar associations with cardiac remodelling and altered function or performance.

Table 3. Clinical evidence to support that vascular calcification is associated with adverse cardiac function and anatomy

Study [year]	Population	n	Age (range or mean±SD)	Calcification	Cardiac outcome	Finding
Diederichsen 2013	Healthy older adults who asymptomatic for hypertension	1825	50-60	CAC	ECG	Having CAC>0 did not increase odds of LVH or strain pattern
Gaibazzi 2014	Symptomatic angina	1117	64±10	CAC	Stress echocardiogram	CAC higher in those with ischemia during stress echo; OR 2.15 (1.48, 3.13)
Guney 2014	Chronic HD	72	44±12	CAC, carotid plaque	Echocardiogram, ECG	QT dispersion and QTd time correlated with carotid plaque score and CAC
Cho 2015	High CV risk older men with preserved ejection fraction	164	73±5	TAC, AAC	Echocardiogram	AAC and TAC correlated with various echocardiographic parameters; ACS associated LV mass
Cho 2017	Aortic valve replacement	47	64±11	CAC, TAC, AAC	Echocardiogram	TAC associated with LV mass pre- & post-operative
Neilson 2015	Uncontrolled hypertension	147	54±10	CAC	Echocardiogram	No association between CAC and LVH

Part 3: Associations of osteoporosis with vascular calcification and stiffness

1. Mechanisms

Bone loss is a feature of ageing and commonly co-exists with vascular calcification in elderly populations [99]. Epidemiological evidence has pointed towards a potential shared development[100]. Recently, much evidence has come to light that the factors known to influence bone mass have direct roles in the development of calcification.

Wingless-related integration site (Wnt)/beta (β)-catenin signalling is a central pathway of bone formation. Wnt & β-catenin are functionally connected elements of a signalling cascade that is necessary for the commencement of osteoblast differentiation and proper bone formation through regulating gene expression, most notably the *RunX2* gene[101,102]. Conditional knockdown of these elements or upregulation in factors known to supress these signalling elements can result in ectopic bone formation or mineralisation of tissue and this has been demonstrated *in vitro* and *in vivo* [103–106]. As such, there is biological and clinical interest into how Wnt/β-catenin signalling elements including its

regulators and suppressors may contribute to the development of vascular calcification given the bone-like appearance and features of vascular calcification.

The differentiation of vascular smooth muscle cells (VSMCs) into bone-like cells capable of laying down bone matrix is a key mechanism of vascular calcification. VSMCs are the cellular components of the normal blood vessel wall. These cells provide structural integrity and can also regulate the diameter of the vessels by contracting and relaxing dynamically in response to vasoactive stimuli. VSMC are unique in that given the appropriate signals they can undergo trans-differentiation into a cell that displays surfaces markers such as RANK and release factors normally attributable to bone cells such as RANKL, osteocalcin and alkaline phosphatase [71,107,108]. Wnt/β-catenin signalling is important in the process of VSMC trans-differentiation. Wnt/β-catenin signalling can promote osteogenesis by directly stimulating *Runx2* gene expression and this process is seen in VSMCs of the arterial wall [109]. It has been demonstrated in a rodent model of vascular calcification that *Runx2* expression is induced and β-catenin is activated by a high-phosphate environment [110]. Furthermore, a specific Wnt signalling molecule, Wnt3a, upregulated osteocalcin expression in VSMCs and promoted calcium deposition in the VSMCs. In cultured cells of the aortic *tunica media* of these rats, β-catenin was activated and *Runx2* mRNA levels correlated with the abundance of β-catenin in the aortic walls. Whilst activation of β-catenin by Wnt induced *Runx2* expression, inhibition of Wnt/β-catenin by natural inhibitors of Wnt signalling such as Dickkopf-1 (DKK-1) and sclerostin, can attenuate *Runx2* induction and thus limit the progression to calcification. Taken together these observations demonstrate that *Runx2* (from VSMC) may mediate the action of Wnt/β-catenin signalling in promoting vascular calcification [Figure 6]. Given the importance of these factors to bone development and skeletal maintenance over the life course, the relevance of bone loss to the development of vascular calcification is of clinical interest. Currently however, there are no clinical data evaluating directly the effect of modulating the Wnt signalling pathway in the context of vascular calcification. Trials examining the anti-sclerostin antibody, romosozumab, have demonstrated efficacy in terms of fracture risk reduction and BMD improvements [111,112]. The trial in men revealed an increase in adjudicated cardiovascular events [n=8/163 (4.9%) in romosozumab treated versus n=2/81 (2.5%) in placebo treated men]. This difference appeared to be driven by a significant difference in the number of cardiac ischaemic events in the romosozumab treated men [n=3/163 (1.8%) versus n=0/81 (0%)] and given the nature of ischaemic events, likely atherosclerotic in origin, we can draw inferences about potentially pro-atherosclerotic effects on sclerostin inhibition. Despite lumbar radiography being conducted on trial participants, no data are yet available on the effect of romosozumab on aortic calcification [111].
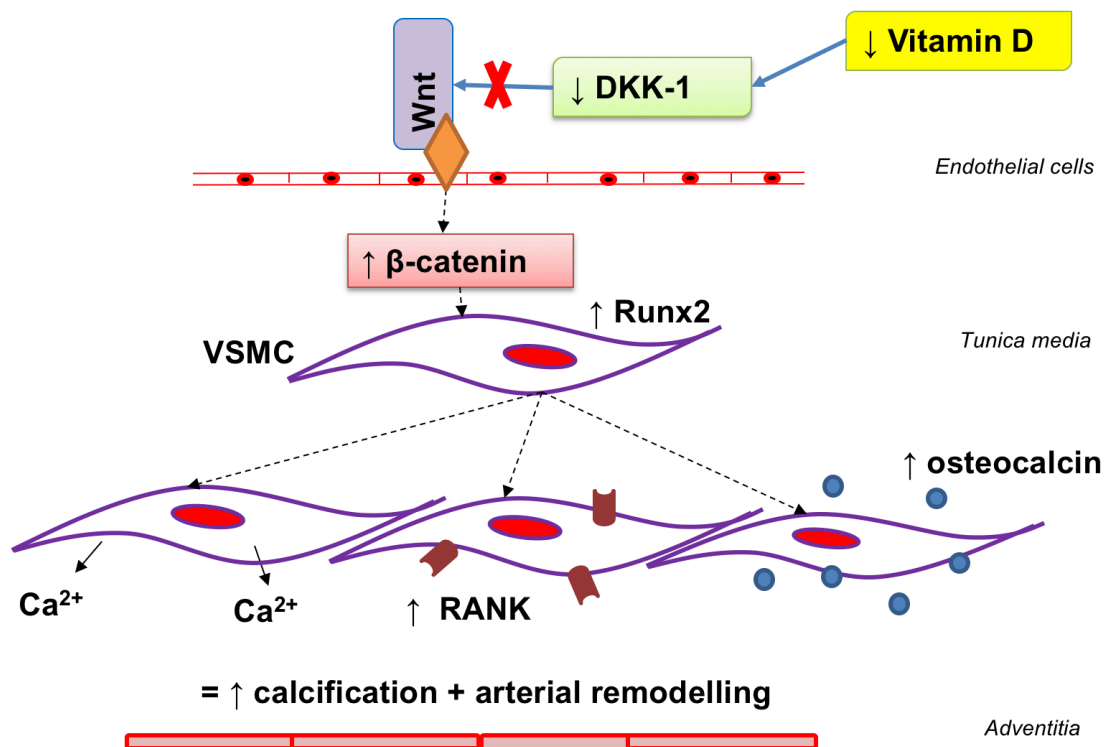
Figure 6. Role of vitamin D, DKK-1 and VSMC in arterial calcification and remodelling. Low vitamin D is common in the elderly and more pronounced with increasing adiposity. Wnt signalling inhibitors are vitamin D regulated elements and thus without natural inhibitors, Wnt is allowed to signal. Intracellularly, VSMC increased the expression of the gene RunX2 which elicits a multitude of effects including increasing calcium efflux from the cell, increasing expression of RANK and increasing the secretion of osteocalcin. The sum total of these process is that the VSMC undergoes trans-differentiation into a bone-like cell capable of laying down bone matrix promoting arterial remodelling and eventual calcification of the vessel.

2. Epidemiological insights into the bone-vascular axis

These observations have been supported in human studies. In a cross-sectional analysis of elderly women who took part in a randomised controlled trial of calcium supplementation for fracture reduction, circulating levels of the Wnt antagonist DKK-1 were evaluated [113]. The study cohort was stratified into quartiles of circulating DKK-1 concentration and the proportion of women with severe AAC on lateral spine radiographs was lowest in the highest quartile of DKK-1, indicating an inverse relationship. Additionally, compared to the highest quartile of DKK-1, the lowest and the second lowest quartiles had approximately two-fold increased likelihood of having any AAC and near two-fold increased likelihood of severe AAC. These associations were independent of important cardiovascular risk factors including previous hospitalisation for vascular disease, renal function and anti-hypertension medication use. In another study of post-menopausal women, DKK-1 was inversely associated with AAC on a 24-point scoring scale and women with a score of one or less (indicating low or no AAC) had significantly less circulating DKK-1. Additionally, women with high pulse wave velocity (a measure of arterial stiffness) had higher levels of sclerostin [114]. Sclerostin is encoded by the *SOST* gene and is produced by osteocytes. Sclerostin has profound anti-anabolic effects on bone formation through inhibition of

elements of the Wnt signalling pathway including low-density lipoprotein receptor related proteins-4, -5 & -6. In a murine model of aortic aneurysm and atherosclerosis, transgenic mice for the *SOST* gene were protected from plaque development and the aortae showed differential expression of proteins known to be involved in the degradation of blood vessel matrix [115]. These observations demonstrate that established modulators of Wnt signalling may protect against the progression of calcification possibly by inhibiting the upregulation of β-catenin activity. This clinical and pre-clinical evidence supports a role for regulators of bone mineral metabolism in the development and progression of vascular calcification and stiffness.

DKK-1 and sclerostin are vitamin D regulated genes, and supplementation appears to modify their serum levels. Therefore, vitamin D may be a clinical link between bone metabolism and vascular calcification and stiffness [Figure 6]. A small sample of young individuals (average age 32 years) with vitamin D deficiency (25-hydroxyvitamin D concentration <20ng/mL) received three once-monthly intramuscular injections of 300,000IU of vitamin D. It was observed that there was a statistically significant difference between pre-treatment and post-treatment values in 25(OH)D levels, parathyroid hormone (PTH) and sclerostin levels demonstrating their responsiveness to vitamin D [116]. In a similar study of older individuals (mean age 61 years), a single intramuscular dose of 300,000IU of vitamin D increased serum levels of DKK-1 and sclerostin and the changes in sclerostin were maintained after three months. Further in regression analysis, only the change in serum vitamin D was found to be a significant predictor of serum DDK-1 levels at three months [117].This would suggest that that vitamin D deficiency may be implicated in adversely affecting the Wnt/β-catenin signalling pathways which consequently provides the conditions for VSMC differentiation and calcification. However, it has yet to be conclusively demonstrated that vitamin D supplementation can modify the potential vascular effects of Wnt/β-catenin signalling pathways including, ultimately, vascular calcification. Given the chronic nature of vascular calcification, it may be unfeasible to test the effect of vitamin D supplementation in a randomised setting. Thus, much literature has been devoted to understanding the effects of vitamin D supplementation on surrogate markers of calcification including endothelial function and arterial stiffness. Several randomised trials have examined the effects of vitamin D supplementation on markers of arterial stiffness across multiple populations including type 2 diabetes [118], hypertension[119] and chronic kidney disease[120] coincidently all these co-morbidities are populations in which aortic calcification is highly prevalent [Table 2]. These clinical data have provided inconsistent evidence and thus the utility, from a clinical and public health perspective, of vitamin D supplementation in improving vascular disease is unknown. Synthesis of this evidence would be of enormous benefit in terms of determining what factor contributed to null/negative findings such as dosing strength and length of supplementation as well as identifying the particular patient groups which may likely benefit most from a specific supplementation regimen. Given the majority of these studies did not enrol individuals with known vitamin D deficiency/insufficiency, future trials specifically recruiting these individuals are needed. Vitamin D supplementation in non-vitamin D deficient populations have proved futile whereas supplementation in deficient populations has led to improved outcomes [121].

The relationships between DDK-1, sclerostin and vitamin D with calcification provide mechanistic insight supporting observations linking low bone mineral density (BMD) and fractures with adverse cardiovascular disease outcomes. In a study of 3,676 healthy older women, it was demonstrated that yearly BMD loss at the femoral neck was greatest in those in the highest quartile of systolic blood pressure (SBP)[36] and in another study of 3,151 healthy older men and women, hypertension was more prevalent in those who had a history of hip fracture (31.3%) compared to those who had not previously fractured (22.5%). In multivariable regression, previous wrist fractures were associated with increased likelihood of hypertension (odds ratio (OR)= 1.48; 95% confidence interval [CI]=1.10, 1.99) though hip fractures were marginally non-significant (1.19; 0.98, 1.45)[29]. These data support our understanding of a shared relationship between bone and vascular disease however, the direction of the relationship (if bone loss precedes vascular disease or vascular disease promotes bone loss) is not understood. Indeed, there appears to be bi-directional relationships between osteoporosis, fracture and cardiovascular disease [Table 1]. In a large study of all 31,936 twins born in Sweden between 1914 and 1994, it was shown that cardiovascular disease increases the risk of fractures. In multivariable models, the hazard ratio (HR) for hip fracture after a diagnosis of heart failure was 4.40 (3.43,5.63); after a stroke, 5.09 (4.18,6.20); after a diagnosis of peripheral atherosclerosis, 3.20 (2.28,4.50); and after an ischemic heart disease event, 2.32 (1.91,2.84) [30]. This finding was replicated in men, where in a large cohort study of 113,600 individuals including 60,637 men, atrial fibrillation was associated with a near doubled risk of incident fractures[35]. Given the orthodox understanding that loss of bone mass occurs concomitantly with the development and progression of aortic calcification; and considering the potential impact of aortic calcification on cardiac function leading to cardiovascular events - it then follows that low bone mass would be associated with poor cardiac function. This has yet to be shown clinically and would enhance our understanding of why cardiovascular diseases are highly prevalent in individuals with low bone mass and in those who have fractured.

3. <u>If we stop bone loss can we reduce vascular disease?</u>

Although negative media attention about the risks of osteoporosis treatment, prescriptions for bisphosphonates in Australia remain high[122]. A meta-analysis of 61 trials examining the effects of bisphosphonates on any cardiovascular outcome identified two studies (totalling n=152 patients) that included a measure of vascular calcification. Bisphosphonates were found to produce a significant reduction in vascular calcification (mean difference between treated and control= -11.52 units [95% CI:-16.51, -6.52; p < 0.01; heterogeneity between studies: I^2=13%])[123]. Despite low statistical heterogeneity, the studies themselves were quite distinct; the larger of the two trials was conducted in 108 Japanese patients with hypercholesterolemia randomised into three treatment groups: atorvastatin (a cholesterol lowering agent) combined with etidronate (a type of bisphosphonate) or either alone [124]. It was found that atorvastatin plus etidronate combination therapy for 12 months significantly reduced both thoracic and abdominal aortic plaques, whereas atorvastatin monotherapy reduced only thoracic aortic plaques and etidronate monotherapy reduced only abdominal aortic plaques. The preferential regression of plaques by etidronate in the abdominal aorta may be explained by the fact that thoracic plaque is more

commonly associated with fatty streaks; whereas abdominal plaques are commonly more calcified. Indeed, in this study calcified abdominal plaques were more prevalent than calcified thoracic plaques [28.7% vs. 13.9% respectively]. The other trial involving bisphosphonates was conducted in a cohort of 50 individuals with chronic kidney disease stages 3-4[125]. It was shown that after 18 months of treatment with alendronate (a type of bisphosphonate) there was no difference in the frequency of progression of vascular calcification between treated and placebo groups (change in Hounsfield units: -24.2 [95% CI: -77.0, 28.6; p=0.4]). Given these studies are relatively small and restricted to patient populations, further research is required to determine effects of bisphosphonates on cardiovascular health.

There is one study that has examined the effect of denosumab on aortic calcification in a randomised controlled trial design. This trial was a secondary evaluation of the larger "Fracture Reduction Evaluation of Denosumab in Osteoporosis Every 6 Months (FREEDOM)" study [126]. Over 7000 women aged 60-90 received denosumab or placebo every six months for 36 months. A subset of women with a mean age of 74 years at baseline [placebo: n=501 and denosumab: n=544] had lateral spine imaging evaluated for AAC. It was demonstrated that, after three years of follow-up, the frequency of progression of AAC did not differ between denosumab [22% with AAC progression] or placebo [22%] groups [p=0.98] [127]. This finding was consistent even when the analysis was restricted to those who had severe AAC at baseline (i.e. those most likely to provide evidence for AAC progression). In this restricted analysis 15% showed progression in denosumab groups versus 19% in placebo treated group. Outside of randomised studies, there also exists a case report of a 57 year old Japanese women with multiple myeloma and chronic kidney disease [stage not reported] who was treated for ten months with denosumab [128]. She developed rapidly progressive vascular and soft tissue calcification to which her physicians attributed to the large doses of vitamin D and calcium she was treated with to correct severe hypocalcaemia secondary to denosumab treatment. These reports, taken together would thus cast doubt on the increasingly accepted bone-vascular axis. It appears that strategies with known effects on bone do not appear to influence vascular calcification biology. Thus, other strategies which known effects on bone and vascular health need to be identified.

Part 4: Associations of sarcopenia with vascular calcification and stiffness

1. Mechanisms potentially linking sarcopenia to vascular calcification and stiffness

Many factors are thought to contribute to age-related declines in muscle mass and quality. Cellular and molecular triggers of muscle loss include myocyte apoptosis, alterations in muscle protein turnover, suboptimal utilisation of dietary amino acids for protein synthesis and impaired satellite cell function and regeneration [40]. These triggers are responsible for oxidative stress, mitochondrial dysfunction, inflammation, hormonal changes, fibre disorganisation and neuromuscular imbalances which can lead to the preferential loss of fast motor units. Disuse and generally low levels of physical activity (which are common in the elderly) are key contributors to these adverse changes in muscle biology[129].

Significantly, inflammation and increased oxidative stress can promote the differentiation of regenerative satellite cells into non-contractile non-functional adipose tissue [so called intra-/inter-muscular adipose tissue (IMAT)] [25]. A higher amount of IMAT therefore increases the load burden of remaining muscle and is independently associated with poor physical performance in older adults [45]. Also, obesity has been associated with inflammation, muscle triglyceride content and serum IL-17 levels as well as the development of early atherosclerosis which provides evidence for shared mechanisms between vascular calcification and muscle loss [130]. Interestingly, fat infiltration into skeletal muscle (one of the features of IMAT) also increases the risk of cardiovascular mortality, suggestive of an interplay between the musculature and vasculature [46] [Table 4]. However, this study did not provide data on the type of cardiovascular mortality and therefore no comment can be made as to the potential association and involvement of IMAT (which may be indicative of a more generalised disease in skeletal muscle) and atherosclerotic vascular disease.

Table 4. Clinical evidence supporting an association between muscle mass and vascular calcification and calcified atherosclerosis

Study [year]	Setting [n]	Age range	Muscle measurement	AAC measurement	Finding
Alexandersen 2006[131]	Denmark, Healthy older men, 168	44-86	Whole body DXA	Lateral spine radiographs, abdominal aorta	Peripheral (appendicular) lean mass negatively associated with aortic calcification severity
Szulc 2012[132]	France, Healthy older men, 1071	20-87	Myostatin	Lateral spine DXA, abdominal aorta	Highest quartile of myostatin associated with lowest odds of AAC; association more robust in men >60
Jensky 2014 [133]	USA, Healthy older adults, 1020	45-84	Abdominal muscle on CT	Abdominal CT (coronary artery and aorta)	Null association between abdominal muscle mass and CAC, TAC and AAC
Idoate 2015[134]	Spain, Institutionalised adults, 42	>80	Abdominal muscle on CT	Abdominal CT (coronary artery)	CAC score did not differ between robust and frail individuals
Wassel 2015[135]	USA, Postmenopausal women, 439	>55	Abdominal muscle on CT	Abdominal CT (coronary artery and aorta)	Greater percentage change in CAC in those with lower abdominal muscle mass
Ko 2016[136]	Korea, Healthy adults, 31108	>18	Whole body bioelectrical impedance	Abdominal CT (coronary artery)	Odds of higher CAC increased with decreasing skeletal muscle index
Idoate 2017[137]	Spain, Institutionalised adults, 42	>80	Abdominal muscle on CT	Abdominal CT (coronary artery)	Extra-coronary calcium scores higher in frail individuals

2. Epidemiological insights into the muscle-vascular axis

Epidemiological evidence suggests that low muscle mass and low muscle quality (poor strength and/or function) increase the risk of cardiovascular mortality[65,66,138,139]. Despite this, there exists few clinical studies on a potential muscle-vascular disease relationship [Table 4]. The first account of an association between muscle (lean) mass and vascular calcification was reported in a modest cohort [n=168] of

middle-aged to elderly men[131]. Body composition was determined by dual-energy x-ray absorptiometry [DXA] and aortic calcification was detected on lateral spine radiographs. It was shown that aortic calcification was negatively correlated with total body lean mass and there was an even more robust correlation with peripheral lean mass considering the peripheral limbs (otherwise described as appendicular lean mass, ALM). In multivariable regression analysis accounting for age, prevalent cardiovascular disease and serum total cholesterol, there was an inverse association between peripheral lean mass and aortic calcification severity (β=-0.153, p=0.049) This observation was subsequently supported by a large cross-sectional study of healthy adult men and women (mean age approximately 40 years)[136]. In this cohort, individuals in the lowest quintile of skeletal muscle mass had the greatest prevalence of coronary artery calcification (CAC) and greatest CAC score. On multivariable regression analysis, every standard deviation decrease in skeletal muscle mass was associated with an approximate 46% increased likelihood of having greater coronary calcium. This association was consistent in individuals with either high (score>100) or low (score≤100) coronary calcium score suggestive that muscle loss may be associated with both the early development and ongoing progression of disease. The amount of muscle mass is strongly determined by myostatin, a protein of the transforming growth factor-β superfamily[140]. Myostatin has also been implicated, pre-clinically, in glucose homeostasis and atherosclerotic/calcific plaque development by promoting mesenchymal stem cells to undergo osteogenic differentiation[141,142]. Complementing the observation that low muscle mass is associated with more vascular calcification; in a large sample of middle-aged men, higher circulating levels of myostatin were associated with lower odds of having AAC, detected via lateral spine assessments on DXA, after adjustment for multiple risk factors[132]. This finding was consistent in a sub-group analysis of men aged over 60 and indeed AAC prevalence was lowest in the highest quartile of serum myostatin relative to the lower three tertiles combined. Interestingly, AAC prevalence was lowest in individuals in the lowest quartile of C-reactive protein (CRP) which is a biomarker of systemic inflammation. This possibly suggests that both increases in inflammation and a lack of muscle synthesis promoters play a role in aortic calcification. Overall, this study provided a potential biological explanation for a muscle-vascular disease association, though it is uncertain whether these factors causatively effect vascular calcification or that these complimentary observations are age-driven associations. Other epidemiological studies in cohorts of men and women have demonstrated null associations between abdominal muscle mass and calcification in various vascular beds including the ascending and descending aorta and coronary arteries [133].Therefore there could be some age-specific effects not noted in earlier studies or, given the younger age of the cohort in question (approximately 64 years), any association between muscle mass and vascular calcification may be less pronounced. This hypothesis is supported by two studies conducted in institutionalised nonagenarians [134,137]. Low muscle mass and fatty infiltration into muscle were characteristic features of the frail individuals in the cohort. Compared to robust (high functioning) individuals, frail individuals (low functioning) had greater CAC scores but this association did not reach statistical significance[134]. However, extra-coronary calcification was significantly higher in frail individuals suggesting that the association between muscle and vascular disease is more pronounced in older age [137]. Other evidence also points to factors relating to muscle function and quality (supporting the frail/robust findings) having an association with vessel disease

[Table 5]. No study has directly examined associations of measures of physical function with aortic calcification. There appears to be more consistent associations with vascular measurements in women than in men. In a multiethnic study of post-menopausal women, abdominal muscle at baseline was associated with longitudinal changes in CAC and this association differed by ethnicity[135]. Despite these observations, no study exists which directly measures muscle mass or muscle quality (strength or function) at a clinically relevant site such as peripheral limbs (important to physical function and independence) in both men and women and determines the association with AAC.

Table 5. Clinical evidence supporting an association between muscle function and vascular calcification, stiffness and calcified atherosclerosis

Study [year]	Setting [n]	Age range	Functional measurement	Vascular measurement	Finding
Saely 2008[143]	Austria, T2D, 746	Not reported	TUG	Coronary stenosis on CT	>50% stenosis was not associated with mobility impairment
Abizanda Soler 2010[144]	Spain, Healthy older adults, 171	>64	TUG	Carotid plaques on Doppler ultrasound	Greater plaque burden associated with slower TUG times
Den Ouden 2013[145]	Netherlands, Healthy older men, 403	73-91	Handgrip, leg extensor strength; SPPB, ADL	Carotid intima-media thickness (cIMT) on ultrasound	Higher cIMT at baseline associated with lower grip strength
Park 2017 [146]	Korea, Healthy adults, 426	19-64	Handgrip strength	cIMT on ultrasound	cIMT was higher in individuals with low grip strength
Shimuzu 2017 [147]	Japan, Hypertensives, 795	60-89	Handgrip strength	cIMT on ultrasound	Greater handgrip strength associated with increased likelihood of greater cIMT in those with high but not low platelet counts
Suwa 2018[148]	Japan, Healthy adults, 1354	35-59	Handgrip strength, sit and reach test, arm flexibility	cIMT on ultrasound	Poor arm flexibility was associated with greater cIMT and poor trunk flexibility was associated with carotid plaque size
Everson-Rose 2018[149]	USA, Healthy older adults, 6490	45-84	Self-reported walking speed	cIMT on ultrasound, Chest CT for coronary artery calcification (CAC)	Greater cIMT at baseline associated with greater declines in walking speed. Those with slower walking speeds had higher CAC

3. Role of exercise in modulating muscle-vascular axis

Compared to the relative lack of efficacy of osteoporosis medications on vascular disease measures; strategies that address poor muscle mass and function have appeared to exert more favourable effects on vascular calcification and stiffness. There is extensive literature examining the role of exercise on sarcopenia and its components [150]. The consistent message is that power-training (or resistance or loading-type exercise) is the best strategy to combat sarcopenia as this type of intervention is sufficient to induce myogenesis in the elderly [151]. Equally there is extensive literature examining the benefits of exercises on vascular function as measured through estimating arterial stiffness [152,153]. Exercise is thought to deliver these vascular adaptions by augmenting NO-dependent vasodilation, which affects arterial function [154]. To date, no study has directly examined the effect of an exercise intervention, in older people on the progression or severity of AAC. This is despite studies determining direct links between muscle mass and aortic calcification[131].

Observational studies have supported interventional trials in that vascular health is better in individuals (including older adults) who engage in higher amounts of physical exertion (structured activities or incidental activities of daily living) [155]. Furthermore, observational studies have illustrated that important determinants of poor muscle mass and physical function, also are independent risk factors for increased arterial stiffness [Table 5]. For example, obesity has independently been associated with increased arterial stiffness and with sarcopenia in older adults [156]. Accordingly, a meta-analysis of weight loss trials has demonstrated that weight reduction can result in significant improvement in a number of arterial parameters [157]. The difficulty with weight loss is that although it may have profound benefits on the vascular system it may lead to unfavourable musculoskeletal outcomes as is seen in patients who have had weight loss surgery [158]. Therefore, a more generalised strategy that targets all systems concurrently would be the ideal strategy to ensure vascular risk reduction whilst maintaining high musculoskeletal functioning. This strategy would thus target the shared risk factors of these conditions [Figure 2]. Currently, no trials exist examining the impact of lifestyle interventions which are have benefits on multiple risk factors, including exercise programmes on advanced atherosclerotic vascular disease. Studies have however, explored micronutrients such as calcium and vitamin D (critical in both the musculoskeletal and vascular systems) in their relation to vascular risk.

Part 5: Calcium-Vitamin D links to vascular calcification and stiffness

Vitamin D and calcium appear to have numerous roles in musculoskeletal ageing and vascular disease. As outlined above, vitamin D responsive genes are critical to osteogenesis and have a role in vascular calcification. Calcium salts (calcium phosphate, calcium carbonate) are the main mineral constituent of bone and appear in ectopic calcification as well. Mineral perturbations, such as those seen in chronic kidney disease and modelled *in vitro* in the setting of high circulating phosphate, are associated with increased mineralisation of vascular tissue. This has resulted in two major themes of clinical interest that have predominated the literature without resolution:

i) If vitamin D responsive elements are responsible for calcification in non-skeletal sites and low vitamin D (either deficiency or insufficiency) is highly prevalent in individuals with calcification – does supplementation of vitamin D result in a decrease in markers of calcification and vascular disease more generally?

ii) If calcium is the predominant component of calcification, do excessively high intakes of calcium (through dietary or supplemental sources) result in excess calcification and vascular disease more generally?

1. Vitamin D

Vitamin D is a pleiotropic steroid hormone whose primary action is to facilitate calcium uptake in the small intestine, fortifying the collagenous fibres. Vitamin D also has a number of non-skeletal effects that may favourably influence the cardiovascular system such as down-regulation of the renin-angiotensin system, enhancing insulin sensitivity and modulating inflammation [56]. The receptor for vitamin D exists on skeletal muscle myocytes and promotes protein synthesis and trophism by negatively regulating myostatin[159]. The vascular endothelium is also responsive to vitamin D. Endothelial cells express the vitamin D receptor and disruption in vitamin D signalling in the presence of calcium increases thrombogenesis, enhances VSMC proliferation and inflammation; and in cardiac myocytes, deletion of vitamin D may promote calcium absorption suggestive of a role in ectopic calcification [160,161]. Importantly, vitamin D has a role in regulating the Wnt/β-catenin signalling pathway. Whilst these pre-clinical data support a role for vitamin D in the pathogenesis of vascular disease, the evidence for a clinical role for vitamin D supplementation in the treatment of vascular diseases is less clear. Hypovitaminosis D (defined as serum calcifediol/25-hydroxyvitamin D (25OHD) concentration below 50nmol/L [=20ng/mL] as according to The Endocrine Society guidelines) has been the subject of intense investigation. Most prospective studies have reported moderate to strong inverse associations between vitamin D concentrations and cardiovascular diseases, serum lipid concentrations, inflammation, glucose metabolism disorders, weight gain, infectious diseases, multiple sclerosis, mood disorders, declining cognitive function, impaired physical functioning, and all-cause mortality [121].

A large body of observational evidence supports a view that there is an inverse relationship between circulating vitamin D and cardiovascular disease and this has been reviewed elsewhere [162]. What has been less established is the therapeutic potential of vitamin D supplementation directly on vascular markers (eg. calcification), but also indirectly on the underlying mechanisms (eg. inflammation). This is despite the general inverse relationship between vitamin D and cardiovascular disease is consistently evident in specific disease states such as heart failure[163] and specific disease manifestations such as increased arterial stiffness[164]. Interventional studies have provided mixed results regarding supplemental effects on disease markers and underlying mechanisms. For example, in a randomised study of over 300 older adults, 12 months of oral 4000 international units (IU)/day of vitamin D_3 did not produce any beneficial effects on blood pressure, heart rate or arterial stiffness (estimated by the augmentation index, stiffness index and reflection index – all markers of the arterial pressure waveform)

[165]. Though, through a secondary analysis of another randomised controlled trial of vitamin D (monthly 100,000IU) on cardiovascular outcomes including the arterial stiffness measures augmentation index (between group difference in change from baseline: -5.7% [95%CI: -10.8, -0.6] and pulse wave velocity [-0.3m/s; -0-0.6, -0.1], it appears that the beneficial effects of vitamin D is limited to those who have underlying vitamin D deficiency[166] . This is consistent with pre-clinical evidence for the role of vitamin D in regulating genes involved in pathogenic pathways of vascular disease. Furthermore, vitamin D_3 supplementation of daily 4000IU for 12 months in older patients with clinical heart failure (an ejection fraction of less than 45%) improved cardiac function (overall approximate 6% improvement in ejection fraction [95%CI=3.20-8.95]) and reversed left ventricular remodelling [167]. It was hypothesised that reductions in inflammation, which is a leading driver of cardiac remodelling and reduced cardiac function, was responsible for these improvements. Vitamin D has immunomodulatory effects, but it has yet to be conclusively demonstrated that vitamin D can improve the inflammatory status in individuals with known cardiovascular disease. Furthermore, given the potential beneficial effect of vitamin D on heart function, no trial to date has examined if vitamin D supplementation can affect the development and/or progression of vascular calcification, something which may precede clinical heart disease such as heart failure or elevated blood pressure.

2. Calcium

There is great biological and clinical debate surrounding the potential adverse cardiovascular effects of calcium including the role of circulating calcium and calcium intake from dietary and supplemental sources. Several systematic reviews have previously been dedicated to the topic [168,169]. At the molecular level, calcium is required for the production of action potentials at the neuromuscular junction for muscle contraction and is an important intracellular signalling molecule and intercellular second messenger for downstream gene transcription. This is particularly relevant regarding the trans-differentiation of VSMC. Given the standard public health message for healthy ageing (which includes musculoskeletal and cardiovascular health) encourages adequate calcium intake from dietary sources; the following will be dedicated to understanding the effect of dietary calcium in vascular disease[170].

A recent meta-analysis proposed that increasing calcium intake through dietary sources is safe and tolerable [168]. Causing much controversy was the publication of a report from a large population study conducted in Sweden in postmenopausal women which concluded that those who consumed more than 1400mg/day of calcium from dietary sources were at an increased risk of all-cause, cardiovascular and myocardial infarction mortality, but not stroke, sparking much commentary [171]. This finding went against conventional wisdom that higher calcium intake from dietary sources is reflective of a health-conscious lifestyle and favourable health outcomes [170]. More recent studies from other countries including Australia have reported contrasting findings to that of the highly publicised Swedish report indicating that there might be ethnic/regional specific effects of dietary calcium on cardiovascular and other mortality outcomes[172]. Furthermore, given postmenopausal accelerated bone loss, the main focus of

bone preservation has focused on women and thus most studies (including the Swedish report) have enrolled only women. Therefore, more studies directly examining potential sex-specific effects of dietary calcium intake are needed.

It is assumed that underlying vessel diseases account for a large proportion of the cardiovascular deaths in these epidemiological studies. There is scant literature regarding the association of dietary calcium on markers of vascular disease. In the Multiethnic Study of Atherosclerosis, higher estimated dietary calcium intake was associated with a decreased risk for developing CAC [relative risk (RR)=0.73(0.57-0.93)] though other studies have suggested there is no association [173,174]. However total calcium intake including from supplemental sources appeared to increase the risk for incident CAC [RR=1.22(1.07-1.39)] [174]. This would suggest that there may be some adverse effect of calcium at the extremes of plausible intake levels; but this has yet to be shown regarding cardiovascular mortality or vascular disease markers including aortic calcification.

Part 6: Conclusion and proposed disease model

This review explored three fundamental features of ageing: muscle loss, bone loss and vascular disease. There are several biologically and clinically plausible connections linking musculoskeletal decline and vascular disease and numerous key research questions are offered throughout this review highlighting areas for further development in our understanding of how these ageing aspects are potentially connected. These research questions can be answered in both interventional and observational settings. Summarising the available evidence, a conceptual disease model is proposed [Figure 7].
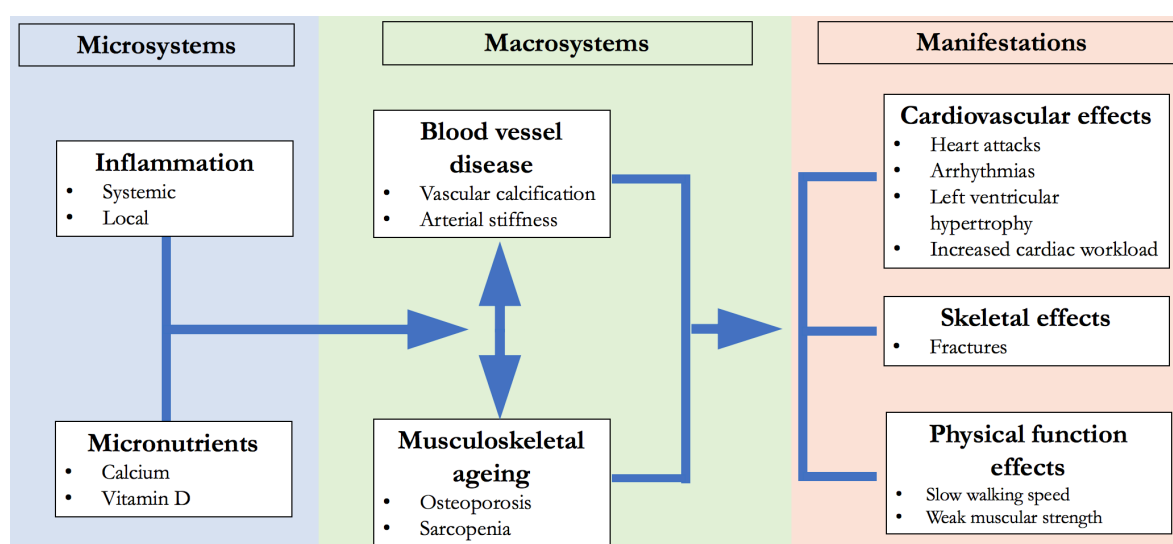
Figure 7. Conceptual disease model. At the microsystemic level, inflammation and the action of small minerals and other micronutrients promote cellular stresses and damage which, if sustained, manifest at the macrosystemic level as tissue loss (bone and muscle) and evidence vascular damage (calcification). The development, progression and acceleration of these disease states may eventually manifest clinically (and indeed become obvious to the individual) evidenced by fractures, poor physical function/functional decline and an inability to perform activities of daily living as well as decreased cardiovascular health.

In this model, central to the manifestations of ageing [poor physical function, adverse skeletal outcomes including fractures and overt cardiovascular disease including heart failure and cardiac events] is the bi-directional nature of the relationship between the musculoskeletal system and the vasculature. Factors that influence the fate of muscle mass and bone mass also appear to influence vessel disease – suggestive of shared biology and fate. The underlying disease state in the microenvironment has direct effects on the musculoskeletal system and the vasculature (the macrosystemic level) and high-quality randomised trials targeting these elements as primary end-points and linking them to hard outcomes are needed to firmly understand disease progression by confirming observational and pre-clinical evidence. Concerning the bone-vascular axis, given the relative lack of efficacy of bone-active medications on calcification there an opportunity for other interventions which target shared risk factors more generally. Calcification is a modifiable process and this improvements through strategies with known effects on bone and muscle (and the risk factors for low bone and muscle) such as exercise (which is well established to promote favourable musculoskeletal) may also promote improvements in calcification. These interventions can be fortified by addressing underlying micronutrient deficiencies which together, may be at the heart of unhealthy ageing.

Funding

No funding was required in undertaking this work.

Conflict of Interest

AJR declares no conflict of interest. DS declares no conflict of interest. PRE declares no conflict of interest.

Ethics approval

No ethics committee approval was required in undertaking this work.

Informed consent

No informed consent was required in undertaking this work as work did not involve any human participants or animals.

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Chapter 2: Associations of muscle mass and function with vascular disease

Chapter 2.1: Introduction

Muscle tissue has emerged in recent years an important endocrine organ capable of secreting a number of cytokines. Thus, interest has increased in understanding contributions of low muscle mass to vascular disease. Inflammatory cytokines which promote muscle atrophy and endothelial dysfunction [26–28] may be implicated in this relationship. Furthermore, as muscle is the largest glucose utilising organ in the body, low muscle mass is understood to reduce insulin sensitivity. This may result in chronic hyperglycaemia which promotes advanced glycation end-product accumulation in the vascular endothelium impacting on arterial contractility [29]. Effects of musculoskeletal decline on the artery can be estimated through measuring arterial pulse wave velocity. There are a number of studies reporting consistent associations with respect to arterial stiffness measures and lower muscle mass and in this Chapter the existing evidence of the association between having low muscle mass and arterial stiffness is synthesised.

However, while arterial stiffness is a useful surrogate marker, vascular calcification represents a more robust marker of vascular disease as calcification indicates established disease [30]. Previous studies have demonstrated that other robust markers of vascular disease such as coronary artery calcification are increased in those with lower muscle mass [31]. No study however has examined associations of

muscle mass and function with abdominal aortic calcification, given calcification at this site is phenotypically different from coronary artery calcification and thus determining associations with other known cardiovascular risk factors will help to elucidate potential biological pathways. Furthermore, it is unknown if an association between muscle mass and function with aortic calcification exists in the general population. This is significant given that aortic calcification can be detected on lateral spine imaging and thus represents an opportunity to assess bone health as well as cardiovascular health[32]. In this Chapter, the association between muscle mass and abdominal aortic calcification was explored in a cohort of older healthy men and women.

During ageing the decline in muscle strength and functional capacity appears to exceed the loss in mass. It is thought that part of this decline in functional capacity is attributable to neuromuscular effects. Neuronal innervation requires adequate vascular supply which may become impaired from chronic structural disease such as calcification. Given, calcification in large vessels is usually indicative of calcification in other smaller vessels, detection of calcification in the aorta (the largest vessel) may be a useful sentinel for generalised disease. In this context, the contribution to vascular disease to muscular strength/functional decline is unknown [33]. Given that muscle strength and physical function can be easily determined in an office setting, there is value in understanding if an association between vascular disease and poor muscle strength and function exists and if these measures could be incorporated into screening or risk assessment tools. This Chapter concludes with

an investigation of the association between muscle strength and physical function and abdominal aortic calcification in a cohort of postmenopausal women.

Chapter 2.2: Systematic Review

Lower muscle tissue is associated with higher pulse wave velocity: A systematic review and meta-analysis of observational study data.

Rodríguez AJ, Karim MN, Srikanth V, Ebeling PR & Scott D

ORIGINAL ARTICLE

WILEY CEPP Clinical and Experimental
Pharmacology and Physiology

Lower muscle tissue is associated with higher pulse wave velocity: A systematic review and meta-analysis of observational study data

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Summary

Muscle loss and arterial stiffness share common risk factors and are commonly seen in the elderly. We aimed to synthesise the existing literature on studies that have examined this association. We searched electronic databases for studies reporting correlations or associations between a measure of muscle tissue and a measure of arterial stiffness. Meta-analysis was conducted using Fisher's Z-transformed r-correlation (r_Z) values. Pooled weighted r_Z and 95% confidence intervals were calculated in an inverse-variance, random-effects model. Heterogeneity was assessed by the inconsistency index (I^2). Study quality was assessed on a checklist using items from validated quality appraisal guidelines. 1195 records identified, 21 satisfied our inclusion criteria totalling 8558 participants with mean age 52±4 years (range 23-74). Most studies reported an inverse relationship between muscle tissue and arterial stiffness. Eight studies had data eligible for meta-analysis. Muscle tissue was inversely associated with pulse wave velocity in healthy individuals [r_Z=−.15 (95% CI −0.24, −0.07); P=.0006; I^2=85%; n=3577] and in any population [r_Z=−.18 (−0.26, −0.10); P<.0001; I^2=81%; n=3930]. In a leave-one-out sensitivity analysis, the results remained unchanged. Lower muscle tissue was associated with arterial stiffness. Studies were limited by cross-sectional design. Cardiovascular risk monitoring may be strengthened by screening for low muscle mass and maintaining muscle mass may be a primary prevention strategy.

KEYWORDS
arterial stiffness, meta-analysis, muscle, pulse wave velocity

1 | INTRODUCTION

Sarcopenia describes the progressive loss of muscle mass and function. Whilst there are numerous consensus definitions to identify those with sarcopenia, it is widely accepted that the loss of muscle tissue during ageing can severely impact a person's health. Already, low muscle tissue has been associated with a number of important outcomes of ageing such as falls, fractures, osteoporosis, decreased quality of life and a loss of independence.[1] Additionally, as muscle is the largest glucose utilising organ in the body, a loss of

muscle tissue may also predispose to an altered glucose metabolism due to a loss in insulin sensitivity.[2] Sarcopenia has also been associated with inflammation and cigarette smoking.[3,4] All the above mentioned factors are well-characterised causal influences on the development of cardiovascular (CV) disease, in particular atherosclerotic vascular disease. These observations have lent support to the hypothesis that sarcopenia may be associated with and is a marker of CV disease.

We now understand that much of the CV disease burden, particularly in older people, may be related to so-called "non-traditional" risk
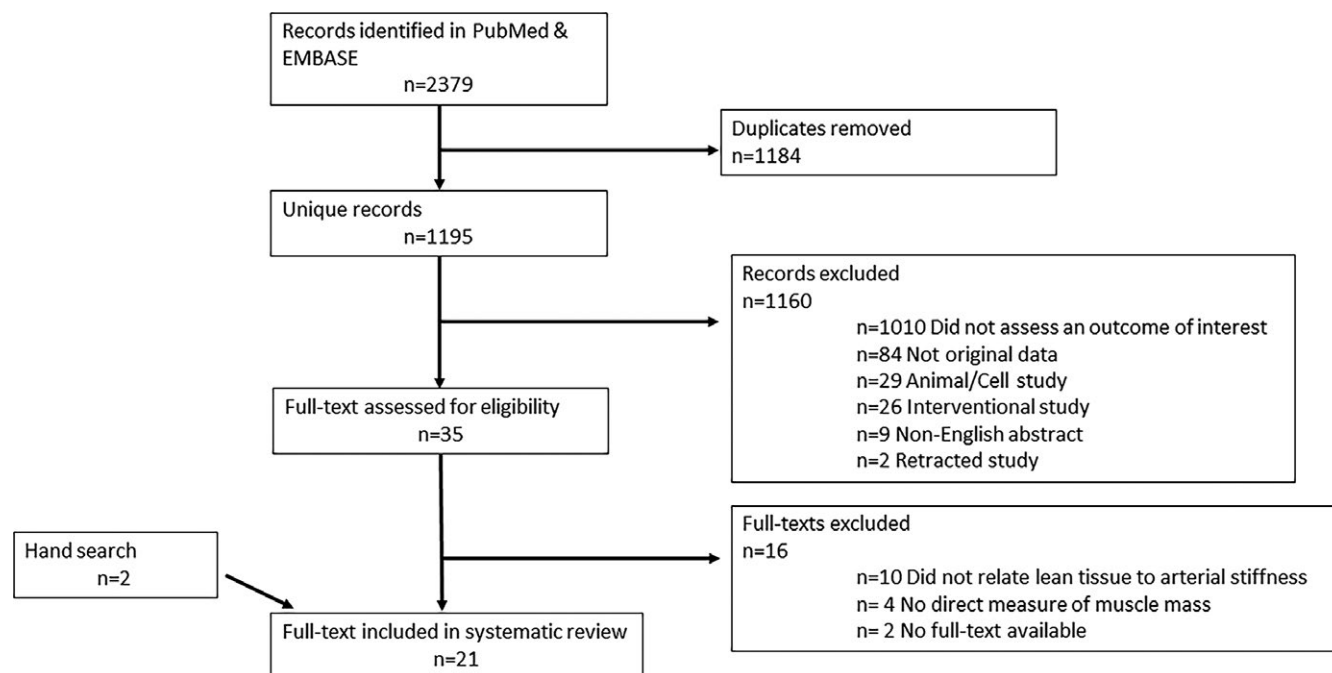
FIGURE 1 Study selection flow diagram

factors such as increased arterial stiffness and pulse pressure.[5] Arterial stiffness describes the elastic properties of a blood vessel and its ability to expand and contract with pressure changes. Heavily calcified or atherosclerotic vessels change their elastic properties such that they become less compliant (stiffer) and are unable to cope effectively with haemodynamic stresses.[6] Increased arterial stiffness augments cardiac workload which may predispose the individual to events such as myocardial infarction or stroke.[7]

The gold standard for arterial stiffness measurement is determination of the carotid-femoral pulse wave velocity (PWV) but there is good agreement between other vascular sites such as the brachial-femoral.[8] In less compliant vessels, the reflected waveform influences (increases) the systolic blood pressure and the extent of this increase is called the augmentation index (Aix). The pulse pressure (PP) describes the difference in the systolic and diastolic blood pressures. Consistently high PP is indicative of stiffness in the large elastic arteries, in particular the aorta. Overall, arterial stiffness is a simple, validated and independent predictor of cardiovascular morbidity and mortality in hypertension, type 2 diabetes, chronic kidney disease and in elderly populations.[8]

Aside from observations that the risk factors for arterial stiffness are also predictive of sarcopenia, there are a number of mechanisms that may directly link the loss of muscle tissue with increased arterial stiffness including inflammatory cytokines, alterations in glucose handling and low physical activity. As such, there is growing observational evidence in humans that low muscle tissue is associated with increased arterial stiffness, however no study exists that has collated this work together and critically analysed the data. Therefore, the aim of this study was to determine if there was an inverse relationship between the amount of muscle tissue and arterial stiffness by way of a systematic review to survey the literature in this area and where possible, meta-analyse data to quantify the relationship.

2 | RESULTS

2.1 | Literature search

Database searching identified 2379 records of which 1184 were duplicates leaving 1195 unique records for title/abstract screening. Following screening, 1160 records were excluded primarily based on the fact the study did not assess an outcome of interest such as PWV, Aix or PP (records excluded on this basis, n=1012) which left 35 records for full-text review. Of these, a further 16 records were excluded mainly because the study did not relate a measure of muscle tissue with arterial stiffness (n=10) leaving 19 records. Snowballing techniques were used in conjunction with hand searching to identify covert literature. At this stage, we identified a further two articles from hand searching reference lists of full-text articles meaning a total of 21 records completely satisfied our inclusion criteria (Figure 1).

2.2 | Characteristics of study design and patient sampling

Twenty studies were cross-sectional[9-28] and one study was prospective in design but still included baseline cross-sectional data.[29] Fifteen studies[10,11,13-15,17-20,22-26,28,29] included a sample of healthy (older or younger) men and women. Three studies sampled participants with type 2 diabetes (T2D),[9,25,26] one study selected participants with an

TABLE 1 Study details and sample demographics

Study & Year	Design	Sample	Country	n	M-F%	Age	BMI (kg/m²)	T2D	SBP	Inclusion-Exclusion criteria
Abbatecola 2012[32]	Prospective	Community dwelling older adults	USA	2272	50-50	73.7	27.2	14.30	134.8	No difficulty walking quarter-mile, climbing 10 steps and performing basic activities of living. Free from life-threatening Illness
Anoop 2015[12]	Cross-sectional	T2D	India	168	65-35	50.3	30.15	100	128.7	n/r or unclear
Benetos 2009[13]	Cross-sectional	Community dwelling older men	France	169	100-0	69.3	27.3	n/r	137	I: >60 years, elegible for DXA, no osteoporosis, no CVD, no atrial fibrillation
Coelho 2015[14]	Cross-sectional	Community dwelling older adults	Brazil	130	0-100	64.3	26.85	4.85	140.4	I: >60 years; E: HRT, psychotropic drugs, CVD, pulmonary disease, psychiatric disease, musculoskeletal disorders, at risk of falls
Convertino 1988[15]	Cross-sectional	Young healthy men	USA	10	100-0	36	n/r	n/r	n/r	E: Taking medications, non-smoking
Corden 2013[16]	Cross-sectional	Community dwelling adults	UK	221	43-57	40.3	25.5	n/r	120.4	E: CVD, meds for hypertension, DM or cholesterol, pregnant or lactating women
Ferreira 2004[17]	Cross-sectional	Community dwelling adults	Netherlands	336	48-52	35.6	24.04	n/r	116.2	n/r or unclear
Hong 2014[18]	Cross-sectional	Community dwelling adults	Korea	452	38-62	53	24.19	n/a	121.9	I: No DM, CVD, hypertension, malignant disease, renal disease, hepatic disease. No meds, alcohol consumption >140 g/week, Hepatitis B or C, herbal medication use in previous 6 months
Kato 2011[19]	Cross-sectional	HD	Japan	161	70-30	61	20.7	n/r	138	E: >80 years, advanced cancer
Kim 2011[20]	Cross-sectional	Community dwelling adults	Korea	510	32-68	53.6	24.38	n/a	121.8	I: No DM, CVD, hypertension, malignant disease, renal disease, hepatic disease. No meds, alcohol consumption >140 g/week, Hepatitis B or C, herbal med use in previous 6 months
Lee 2014[21]	Cross-sectional	Community dwelling older adults	Korea	428	42-58	70.9	24	n/r	132	E: patients with incomplete testing records
Loenneke 2013[22]	Cross-sectional	Community dwelling adults	USA	27	48-52	23	24.4	n/r	118	I: non-smoker, no CVD or metabolic disturbance
Ochi 2010[23]	Cross-sectional	Community dwelling middle age to older adults	Japan	496	100-0	n/r	n/r	n/r	n/r	I: free of cerebrovascular events
Okabe 2004[24]	Cross-sectional	OT in-patients	Japan	24	58-42	n/r	n/r	n/r	n/r	I: Had cerebrovascular event in previous 3 months, physically stable
Sampaio 2014[25]	Cross-sectional	Community dwelling older adults	Japan	175	48-52	73.6	23.02	11.7	^41.30%	I: >65 years, independent. E: mild cognitive impairment, uncontrolled CVD/metabolic disease, orthopaedic conditions, risk of falls
Sanada 2012[26]	Cross-sectional	Community dwelling older women	Japan	533	0-100	30-84	n/a	n/r	122.3	I: Free of CVD, not taking beta-blockers or steroids or HRT. Free of chronic disease. Mod PA

(Continues)

TABLE 1 (Continued)

Study & Year	Design	Sample	Country	n	M-F%	Age	BMI (kg/m²)	T2D	SBP	Inclusion-Exclusion criteria
Sanada 2010[27]	Cross-sectional	Community dwelling older adults	Japan	959	50-50	28	21.9	n/r	125.9	I: active and free of CVD. E: use of beta-blockers, steroids, HRT
Snijder 2009[28]	Cross-sectional	Older men and women with and without diabetes	Netherlands	456	50-50	68.3	26.15	18.7	n/r	n/r or unclear
Snijder 2004[29]	Cross-sectional	Older men and women with and without diabetes	Netherlands	488	50-50	69	26.4	22	140.7	n/r or unclear
Tabara 2009[30]	Cross-sectional	ABI>0.9	Japan	407	40-60	68	23.18	11.1	128.9	n/r or unclear
Wohlfahrt 2015[31]	Cross-sectional	Healthy, non-obese	USA	136	44-56	45	24.3	n/a	114	I: Age 30-65 years BMI 18.5-29.9 kg/m² E: liver disease, ESRD, lung cancer, cancer, hypertension, T2D, pregnancy, lactating, excessive adiposity, metabolic diseases, previous CVD event, smokers, Type 1 DM, involuntary weight loss in last 2 years, bariatric surgery

^Percentage of individuals with SBP >140 mmHg.

ankle-brachial index >0.9;[27] one study selected participants on maintenance haemodialysis (HD);[16] one study selected young healthy astronaut trainees[12] and one study was conducted in patients undergoing occupational therapy for hemiparesis.[21] Most studies were conducted in Japanese or Korean populations.[15-18,20-24,27] Other studies were conducted in USA,[12,19,28,29] Brazilian,[11] Indian,[9] French,[10] British[13] and Dutch[14,25,26] populations (Table 1).

2.3 | Quality assessment

All but one study[12] achieved a quality score of at least 5 (out of 10). Six studies were deemed high quality achieving a score greater than 8.[10,13,15,22,28,29] Commonly reported items were study aims and outcomes; important confounding factors such as age, sex and BMI; and measuring muscle tissue using validated methods. Inclusion/exclusion criteria, sample generalisability, adjustment analyses for multiple confounding factors, using validated methods to quantify arterial stiffness and identifying study limitations were variously reported (Table S1).

2.4 | Sample demographics

Study populations varied from a minimum of 10[12] to 2272.[29] The average number of participants per study across the included literature was 387 and the total number of participants across all 21 included studies was 8558. Three studies included only men[10,12,20] and two studies included only women[11,23] and there was an average of 51% males per study and 49% females per study. The lowest mean age was 23 years[19] and the highest was 74 years.[29] Mean age for the study sample was not reported in three studies[20,21,23] but these reported age ranges. Mean BMI ranged from a minimum 21.9 kg/m²[24] to a maximum of 30.2 kg/m².[9] Mean BMI for the sample was not reported in four studies[12,20,21,23] (Table 1).

2.5 | Assessment of muscle tissue

Most studies reported a measure of muscle mass[9-15,17,18,21-26,28,29] (Table 3). Five studies reported measure of muscle cross-sectional area.[12,16,19,20,27] Muscle tissue was predominantly measured as either a whole body assessment[10-13,15,21,22,29] or as an appendicular assessment.[9,14,17,18,23,24,28] Other studies measured muscle tissue or cross-sectional area in specific body regions such as the thigh[16,19,20,27] or trunk.[25,26,29] Most studies used radiological imaging to determine lean tissue including dual-energy x-ray absorptiometry (DXA),[10,14,15,17,21,23-26,28,29] computed tomography (CT),[12,16,17,20,27] or peripheral quantitative computed tomography (pQCT).[19] Other studies utilised bioelectrical impedance analysis (BIA).[9,11,13,18,22] Assessment of sarcopenia was performed in seven studies[11,15,17,22-24,29] where in all cases the operational definition consisted of skeletal/appendicular muscle mass relative to height-squared. Six of these studies utilised a cut-point for the definition, including the previously published Baumgartner[30] definition[17,29] and other user defined cut-points from a reference population[11,15,23,24] (Table 2).

TABLE 2 Assessment of muscle tissue

Study	Parameter	Region	Modality	Device	Sarcopenia defined	Cut-point
Abbatecola[32]	Muscle mass	Whole body	DXA	Hologic	ASM/h^2	Baumgartner[30]
Anoop 2015[12]	Muscle mass	Appendicular	BIA	Tanita	n/d	n/a
Benetos 2009[13]	Muscle mass	Whole body	DXA	Hologic	n/d	n/a
Coelho 2015[14]	Muscle mass	Whole body	BIA	Tanita	SMI (kg/m^2)	Lower tertile (<15.68 kg/m^2)
Convertino 1988[15]	Muscle mass and CSA	Whole body, calf	Hydrostatic weighing, CT	Siemans	n/d	n/a
Corden 2013[16]	Muscle mass	Whole body	BIA	InBody	n/d	n/a
Ferreira 2004[17]	Muscle mass	Trunk and appendicular	DXA	Hologic	n/d	n/a
Hong 2014[18]	Muscle mass	Whole body and legs	DXA	Hologic	SMI (kg/m^2)	1SD below reference
Kato 2011[19]	Muscle CSA	Thigh	CT	n/r	n/d	n/a
Kim 2011[20]	Muscle mass	Appendicular and mid-thigh	DXA, CT	Hologic	ASM/h^2	Baumgartner[30]
Lee 2014[21]	Muscle mass	Appendicular	BIA	Biospace	n/d	n/a
Loenneke 2013[22]	Muscle CSA	Femoral	pQCT	Stratec	n/d	n/a
Ochi 2010[23]	Muscle CSA	Femoral	CT	GE	n/d	n/a
Okabe 2004[24]	Muscle mass	Whole body and legs	DXA	Hologic	n/d	n/a
Sampaio 2014[25]	Muscle mass	Absolute	BIA	n/r	SMI (kg/m^2)	n/r
Sanada 2012[26]	Muscle mass	Appendicular	DXA	Hologic	SMI (kg/m^2)	*Below a median of 6.70
Sanada 2010[27]	Muscle mass	Appendicular	DXA	Hologic	SMI (kg/m^2)	*Class 1=7.77-6.12; Class 2=6.87-5.46
Snijder 2009[28]	Lean mass	Trunk and Leg	DXA	Hologic	n/d	n/a
Snijder 2004[29]	Lean mass	Trunk and Leg	DXA	Hologic	n/d	n/a
Tabara 2009[30]	Muscle CSA	Femoral	CT	GE	n/d	n/a
Wohlfahrt 2015[31]	Lean mass	Trunk and appendicular	DXA	GE Lunar	n/d	n/a

*Used a Japanese reference population.

2.6 | Assessment of arterial stiffness

The majority of studies assessed PWV[9,10,13-17,20-29] other studies assessed PP,[11,19] Aix[18,19] and one study assessed leg compliance[12] (Table 3). For studies assessing PWV, eight studies assessed brachial-ankle PWV;[15-17,20,21,23,24,27] seven studies assessed carotid-femoral PWV[9,10,14,25,26,28,29] and one study assessed carotid-ankle PWV.[22]

2.7 | Relationship between muscle tissue and arterial stiffness

Thirteen studies reported on the relationship between measures of muscle tissue and measures of arterial stiffness[9-16,19-21,28,29] (Table 4). The majority of these studies reported an r-correlation statistic to describe the strength and direction of the relationship between the amount of muscle tissue and the measure of arterial stiffness.[9,11-13,15,16,19-21,28,29] Two studies provided a β-statistic to describe the direction of the relationship between muscle tissue and arterial stiffness.[10,11,14] For r-correlations relating muscle to PWV, the magnitude of the statistic ranged from −.02 (for the correlation between arm mass and PWV)[9] and −.4795 (trunk mass and PWV)[28] in unadjusted analyses. In adjusted analyses, r-correlations ranged from −.0082 (arm mass in women and PWV)[29] adjusting for multiple confounding factors, and −.67 (whole body muscle mass) adjusting for age, bone mineral content, fat mass and intramuscular fat.[21] In studies reporting r-correlations for PP one study reported a negative correlation[11] and another reported a positive correlation.[19] Calf muscle area was negatively correlated with Aix after adjustment of height and sex in one study.[19] In the study evaluating leg compliance, whole body lean mass was positively correlated with compliance whilst calf muscle area was negatively correlated.[12] For studies reporting a β-statistic, all three studies reported a negative relationship between muscle mass, peripheral muscle mass and PWV and between skeletal muscle index (SMI) and PP.[10,11,14] Three studies

TABLE 3 Assessment of arterial stiffness

Study	Parameter	Vascular site	Method	Device
Abbatecola[32]	PWV	Carotid-femoral	Doppler transcutaneous probes	Park Medical Electronics
Anoop 2015[12]	PWV	Carotid-femoral	Applanation tonometry	SphygmoCor
Benetos 2009[13]	PWV	Carotid-femoral	Applanation tonometry	PulsePen
Coelho 2015[14]	PP	n/a	Oscillometric	Microlife
Convertino 1988[15]	Leg Compliance*	Calf	Oscillometric	n/r
Corden 2013[16]	PWV	Aortic arch	Flow waveform analysis	CMRtools Cardiovascular Imaging Solutions
Ferreira 2004[17]	PWV	Carotid-femoral	Volume-plethysmographic apparatus	Komaki
Hong 2014[18]	PWV	Brachial-ankle	Volume-plethysmographic apparatus	Komaki
Kato 2011[19]	PWV	Brachial-ankle	Oscillometric	Fukuda Denshi
Kim 2011[20]	PWV	Brachial-ankle	Volume-plethysmographic apparatus	Komaki
Lee 2014[21]	Aix	Radial	Pulse Wave Analysis	Omron
Loenneke 2013[22]	Aix, PP	Radial	Applanation tonometry	SphygmoCor
Ochi 2010[23]	PWV	Brachial-ankle	Volume plethysmographic apparatus	Omron
Okabe 2004[24]	PWV	Brachial-ankle	Volume-plethysmographic apparatus	Komaki
Sampaio 2014[25]	PWV	Carotid-ankle	Oscillometric	Fukuda Denshi
Sanada 2012[26]	PWV	Brachial-ankle	Volume-plethysmographic apparatus	Komaki
Sanada 2010[27]	PWV	Brachial-ankle	Volume-plethysmographic apparatus	Komaki
Snijder 2009[28]	PWV	Carotid-femoral	Oscillometric	Collin Press-Mate
Snijder 2004[29]	PWV	Carotid-femoral	Oscillometric	Collin Press-Mate
Tabara 2009[30]	PWV	Brachial-ankle	Oscillometric	Omron
Wohlfahrt 2015[31]	PWV	Carotid-femoral	Applanation tonometry	SphygmoCor

*Defined as % change in blood volume relative to change in blood pressure.

determined that the relationships were significant in men and not in women[9,20,29] and another study determined thigh muscle area was significant in a sample of middle aged to elderly men but was non-significant when the group was stratified into those with type 2 diabetes mellitus and those that did not.[20] Overall, only four studies did not report a significant relationship[10,12-14] between muscle tissue and arterial stiffness. There was a consistent trend across all studies for measures of muscle tissue at various sites in the body to be negatively related to arterial stiffness (mostly PWV) in unadjusted and multiple adjusted analyses.

2.8 | Meta-analysis of studies reporting correlation statistics

The most complete and consistent data related to the correlation between muscle tissue with PWV which included data from eight studies.[9,13,15,16,20,21,28,29] These data were synthesised into a meta-analysis model. In studies enrolling only healthy participants, muscle tissue was inversely and significantly correlated with PWV (r_Z=−.15

and 95% confidence interval=−0.24, −0.07; P=.0006, n=3577, five studies) with substantial heterogeneity (I^2=85%) (Figure 2). When data was included from studies enrolling participants from specific patient groups, the results remained similar PWV (r_Z=−.18; −0.26, −0.10; P<.0001, n=3930, eight studies; I^2=81%) (Figure 3). In sensitivity analyses (Table 5), considering only studies with a mean sample age of ≥60 years there was data available from two studies[20,29] and there was a significant negative relationship between muscle tissue and PWV (r_Z=−.12; 95% CI −0.22, −0.01; P=.02, I^2=85%). Similarly, in studies whose mean sample age was <60 years, there was present a significant negative relationship between muscle tissue and PWV (r_Z=−.22; 95% CI −0.35, −0.09; P=.0009, I^2=72%). When the studies were stratified according the imaging modality employed, results were similar whether DXA (r_Z=−.19; 95% CI −0.32, −0.07; P=.002, I^2=89%) or other modalities (r_Z=−.16; 95% CI −0.22, −0.10; P<.0001, I^2=42%, fixed model applied) were used. Finally, in order to examine the potential effect the site of muscle tissue assessment, we stratified the studies according to whether the whole body muscle tissue was assessed (r_Z=−.11; 95% CI −0.20, −0.02; P=.02, I^2=82%) or regional muscle

tissue (r_z=−.24; 95% CI −0.34, −0.13; P<.0001, I^2=59%) and found results similar to other analyses. Finally, we performed a leave-one-out sensitivity analysis and omission of any one study did not alter the final result (Table 6).

3 | DISCUSSION

Sarcopenia, the progressive loss of muscle mass and function, has been associated with CV disease. Arterial stiffness is emerging as an

TABLE 4 Correlations and associations between lean tissue and arterial stiffness

Study	Sample	n	Lean tissue	Outcome	r	R^2	β	P	Adjustments	Meta-analysis
Anoop	Female	58	Right leg	PWV	−.21	n/r	n/r	0	Unadjusted	*
			Left leg	PWV	−.23	n/r	n/r	0	Unadjusted	
			Right arm	PWV	−.07	n/r	n/r	.55	Unadjusted	
			Left arm	PWV	−.02	n/r	n/r	.56	Unadjusted	
			Trunk	PWV	−.04	n/r	n/r	.74	Unadjusted	
	Male	110	Right leg	PWV	−.16	n/r	n/r	.09	Unadjusted	*
			Left leg	PWV	−.18	n/r	n/r	.05	Unadjusted	
			Right arm	PWV	−.18	n/r	n/r	.05	Unadjusted	
			Left arm	PWV	−.18	n/r	n/r	.04	Unadjusted	
			Trunk	PWV	−.19	n/r	n/r	.04	Unadjusted	
Benetos		169	Lean mass	PWV	n/r	n/r	−.25	.36	Age, fat mass (kg), BMD	
Coelho		130	SMI	PP	−.7	.49	−.226	.05	Unadjusted	
			SMI	PP	−.417	.174	.98	n/s	Age, metabolic syndrome, smoking, HTN, DM, height, physical function	
Convertino		10	Lean mass	Leg C	.39	n/r	n/r	.26	Unadjusted	
			Calf CSA	Leg C	−.61	.04	n/r	.06	Unadjusted	
Corden		221	LMI	PWV	−.07	n/r	n/r	.191	Age, sex, MAP, %body fat	*
Hong		552	SMI	PWV	−.16	n/r	n/r	<.001	Age	*
Ferriera		336	PLM	PWV		n/r	−.37	n/r	Sex, height, MAP, body composition, VO$_2$max, Total/HDL, TG. HbA1c, HR	
Kato	All	161	TMA	PWV	−.18	n/r	n/r	<.05	Unadjusted	*
	non-DM	127	TMA	PWV	−.18	n/r	n/r	.09	Unadjusted	**
	DM	34	TMA	PWV	−.24	n/r	n/r	.24	Unadjusted	**
Loennecke		27	Muscle CSA	PP	.166	n/r	n/r	.428	Height, sex	
			Muscle CSA	Aix	−.489	n/r	n/r	.013	Height, sex	
Ochi	Men	496	Calf CSA	PWV	−.34	n/r	n/r	<.001	Weight	*
	Women		Calf CSA	PWV	−.09	n/r	n/r	.1	Weight	*
Okabe		24	Muscle mass	baPWV	−.67	n/r	n/r	<.01	Age, BMC, fat mass, IMAT	*
				faPWV	−.627	n/r	n/r	<.01	Age, BMC, fat mass, IMAT	
Wohlfarht		136	Arm	PWV	−.424	.18	n/r	<.05	Unadjusted	
			Leg	PWV	−.4	.16	n/r	n/s	Unadjusted	*
			Trunk	PWV	−.4795	.23	n/r	<.01	Unadjusted	
			Total	PWV	−.447	.2	n/r	<.05	Unadjusted	

TABLE 4 (Continued)

Study	Sample	n	Lean tissue	Outcome	r	R^2	β	P	Adjustments	Meta-analysis
Abbetecola	Men	2272	SI	PWV	−.09	n/r	n/r	.01	Age, diabetes, site, height, BMI, SBP, PAD, CHD, smoking, IL-6, physical activity, race,	
			Arm	PWV	−.106	n/r	n/r	.0005	Age, diabetes, site, height, BMI, SBP, PAD, CHD, smoking, IL-6, physical activity, race,	*
			Leg	PWV	−.217	n/r	n/r	.0003	Age, diabetes, site, height, BMI, SBP, PAD, CHD, smoking, IL-6, physical activity, race,	
	Women		Arm	PWV	−.0082	n/r	n/r	.6849	Age, diabetes, site, height, BMI, SBP, PAD, CHD, smoking, IL-6, physical activity, race,	*

n.b. data shown in italics were sourced from authors or calculated from published data. * indicates if the study had data included in meta-analysis. ** indicates data was treated separately in meta-analysis. Abbreviations: Aix, augmentation index; ba, brachial-ankle; BMC, bone mineral content; BMD, bone mineral density; BMI, body mass index; β, Beta coefficient; CHD, coronary heart disease; CSA, cross-sectional area; DM, diabetes mellitus; fa, femoral-ankle; HbA1c, glycated haemoglobin; HR, heart rate; HTN, hypertension; IL-6, interleukin-6; IMAT, intramuscular fat; Leg C, leg compliance; LMI, lean mass index, MAP, mean arterial pressure; n/r not reported; PAD, peripheral artery disease; PLM, peripheral lean mass; PP, pulse pressure; PWV, pulse wave velocity; r, r-correlation coefficient; R^2, R-squared correlation coefficient; SMI, skeletal muscle index; TG, triglycerides; TMA, thigh muscle area.

Study or Subgroup	Fisher transformed r-correlation	SE	Weight	Fisher transformed r-correlation IV, Random, 95% CI
Wolhfarht 2015	-0.42365	0.086711	10.8%	-0.42 [-0.59, -0.25]
Ochi 2010 (men)	-0.35409	0.075378	12.0%	-0.35 [-0.50, -0.21]
Hong 2014	-0.16139	0.042679	15.7%	-0.16 [-0.25, -0.08]
Abbatecola 2012 (men)	-0.09024	0.02739	17.2%	-0.09 [-0.14, -0.04]
Ochi 2010 (women)	-0.09024	0.056433	14.2%	-0.09 [-0.20, 0.02]
Corden 2013	-0.07011	0.067729	12.9%	-0.07 [-0.20, 0.06]
Abbatecola 2012 (women)	-0.0082	0.02739	17.2%	-0.01 [-0.06, 0.05]
Total (95% CI)			100.0%	-0.15 [-0.24, -0.07]

Heterogeneity: Tau² = 0.01; Chi² = 38.81, df = 6 (P < 0.00001); I² = 85%
Test for overall effect: Z = 3.43 (P = 0.0006)

FIGURE 2 Pooled Fisher's transformed r-correlation for the association between muscle tissue and pulse wave velocity in healthy individuals

important CV risk factor and given the common risk factors between sarcopenia and arterial stiffness observational studies have demonstrated the concomitant incidence of low muscle mass with increased arterial stiffness. This systematic review and meta-analysis collated results from observational studies which collectively demonstrate that low muscle tissue is consistently associated with increased arterial stiffness. Whilst heterogeneity in terms of study design, population samples and methodology may bias and limit the interpretation of the quantitative meta-analysis component of this review; given the consistent inverse relationship seen across all studies included in this review, there is a biological interaction which may not necessarily be

captured statistically due to heterogeneity. For this reason, the present study adds value to the literature as it highlights the need to develop high quality prospective studies conducted in healthy populations using gold-standard techniques to determine if low muscle tissue is both predictive and causative of arterial stiffness.

The causal biological link between low muscle tissue and increased arterial stiffness is only assumed given these data were largely cross-sectional (only one study identified was longitudinal).[29] Therefore, the possible mechanisms by which low muscle tissue may contribute to increased arterial stiffness are unclear. It has also been suggested that increased arterial stiffness precedes the loss of muscle tissue as

Study or Subgroup	Fisher transformed r-correlation	SE	Weight	Fisher transformed r-correlation IV, Random, 95% CI	Fisher transformed r-correlation IV, Random, 95% CI
Okabe 2004	-0.81074	0.218218	2.7%	-0.81 [-1.24, -0.38]	
Wolhfarht 2015	-0.42365	0.086711	8.3%	-0.42 [-0.59, -0.25]	
Ochi 2010 (men)	-0.35409	0.075378	9.2%	-0.35 [-0.50, -0.21]	
Anoop 2015 (men)	-0.21317	0.13484	5.4%	-0.21 [-0.48, 0.05]	
Kato 2011 (All)	-0.18198	0.079556	8.9%	-0.18 [-0.34, -0.03]	
Anoop 2015 (women)	-0.16139	0.096674	7.6%	-0.16 [-0.35, 0.03]	
Hong 2014	-0.16139	0.042679	11.8%	-0.16 [-0.25, -0.08]	
Ochi 2010 (women)	-0.09024	0.056433	10.7%	-0.09 [-0.20, 0.02]	
Abbatecola 2012 (men)	-0.09024	0.02739	12.8%	-0.09 [-0.14, -0.04]	
Corden 2013	-0.07011	0.067729	9.8%	-0.07 [-0.20, 0.06]	
Abbatecola 2012 (women)	-0.0082	0.02739	12.8%	-0.01 [-0.06, 0.05]	
Total (95% CI)			**100.0%**	**-0.18 [-0.26, -0.10]**	

Heterogeneity: Tau² = 0.01; Chi² = 51.66, df = 10 (P < 0.00001); I² = 81%
Test for overall effect: Z = 4.41 (P < 0.0001)

-1   -0.5   0   0.5   1
Lower Muscle Mass   Higher Muscle Mass

FIGURE 3 Pooled Fisher's transformed *r*-correlation for the association between muscle tissue and pulse wave velocity in any population (including individuals with type 2 diabetes and renal impairment)

heavily calcified and stiff blood vessels may restrict nutrient supply to muscle tissue causing its atrophy. However, there exist a number of pathways common to both muscle loss and increased arterial stiffness including insulin resistance, oxidative stress and inflammation which may offer mechanism insights.[31]

Muscle is the largest site of glucose disposal and its loss can impair glucose metabolism which may result in insulin insensitivity/resistance.[32] Several human studies suggest that impaired glucose tolerance and insulin resistance adversely affect the mechanical properties of arteries.[33] The mechanisms that lead to adverse vascular changes are thought to occur through oxidative stress and endothelial damage. In a Korean cohort, PWV positively correlated with insulin and had higher concentrations of isoprostane 8-epi PGF2α (an oxidative stress marker) in those with impaired fasting glucose.[34] Further, in a hypertensive older adult sample, statin therapy improved the oxidative markers *N*-(epsilon)-carboxymethyl-lysine, Von Willebrand factor and copper/zinc-containing superoxide dismutase. These improvements were correlated with improvements in brachial-ankle PWV suggesting that oxidative stress precedes increases in arterial stiffness possibly by increasing reactive oxide species.[35] Taken together, these studies indicate that impaired glucose metabolism and alterations in oxidative stress at the vascular endothelium directly contribute to vessel compliance and offer a link between how muscle biology may influence the development of arterial stiffness.

Chronic inflammation is another well described mechanism of muscle loss. Inflammatory mediators such as interleukin-6 (IL-6), tumour necrosis factor-α and C-reactive protein can directly affect muscle catabolism and may also act indirectly by lowering growth hormones.[36] These inflammatory pathways are shared in the pathogenesis of arterial stiffening. Aortic PWV correlated positively with a number of inflammatory markers such as IL-6, transforming growth factor-β1, and white blood cell count in dialysis patients.[37] Further, osteocalcin, a marker of bone formation produced by osteoblasts, was negatively correlated with aortic PWV after multiple regression. Given osteocalcin's role in bone remodelling, this finding suggests bone biology may influence arterial stiffness and indeed elevated arterial stiffness has been demonstrated in postmenopausal women with osteoporosis.[38]

A number of important limitations of the included studies could contribute to the risk of bias. Firstly, the majority of studies were cross-sectional in design. Given that several of these did not adjust for multiple confounders,[9,16,28] there may be residual confounding in our analysis. Secondly, some studies had small sample sizes (e.g. n=10[12]) or the cohort was from a specific patient population (e.g. hemiparesis inpatients[21]) and thus their results may not be generalisable. Thirdly, the use of medications was poorly reported and this may potentially impact on blood pressure, blood lipid profile and other risk factors of arterial stiffening. Few studies reported on the amount of objectively measured physical activity undertaken by study participants. This is significant because physical activity is important in maintaining muscle mass and decreasing arterial stiffness by augmenting oxidative stress markers, particularly in older age.[39,40] Finally, for stiffness assessment some studies used oscillometric methods and others employed tonometric methods and similarly there were different methods used to quantify muscle tissue (e.g. DXA and CT). There is good agreement between these methods to quantify both arterial stiffness[41] and muscle tissue[42] though these different methods employed across this literature reflects the lack of a standardised approach to measuring arterial compliance and muscle tissue. In attempt to overcome this potential limitation, our sensitivity analyses found similar results when studies were stratified by imaging modality.

The limitations of the present study were that we used aggregate data from a small number of studies. This limited our ability to perform stratified analyses from individual patient data according important confounding factors rather than study-level variables. We also grouped together a heterogeneous sample of studies (Japanese, European and North American populations). Overall, we attempted to overcome the obvious heterogeneity in study methods by employing random-effects models which offer more conservative effect estimates and in performing a number of sensitivity analyses restricting each analysis to studies of similar methodology or population sample. Specifically, our study may be limited by crude measures of body composition which may not accurately reflect the degree of muscle loss and disability burden across diverse populations.[43] Most data were from cross-sectional studies which do not imply causation. We did not restrict our search criteria to exclude interventional studies or other observational studies. Thus, our study highlights the need for more prospective investigations as cross-sectional relationships do

TABLE 5 Sensitivity analyses

Stratification	Studies (n)	Effect	95% CI	P	I^2
Age (years)					
Mean age ≥60	2 (2768)	−0.12	−0.22, −0.01	.02	85
Mean age <60	5 (1162)	−0.22	−0.35, −0.09	.0009	72
Imaging modality					
DXA	4 (2884)	−0.19	−0.32, −0.07	.002	89
Non-DXA	4 (1046)	−0.16	−0.22, −0.10	<.0001	42
Muscle tissue assessment					
Regional	4 (961)	−0.24	−0.34, −0.13	<.0001	59
Whole body	4 (2969)	−0.11	−0.20, −0.02	.02	82

TABLE 6 Leave-one-out sensitivity analysis

Study omitted	n	Fisher transformed r-correlation [95% CI]	P
Okabe 2004	3906	−.16 [−0.23, −0.08]	<.0001
Wolhfarht 2015	3794	−.15 [−0.22, −0.08]	<.0001
Ochi 2010	3434	−.17 [−0.25, −0.08]	<.0001
Anoop 2015	3762	−.18 [−0.27, −0.09]	<.0001
Kato 2011	3769	−.18 [−0.26, −0.09]	<.0001
Hong 2014	3508	−.18 [−0.27, −0.09]	<.0001
Abbatecola 2012	1658	−.20 [−0.30, −0.10]	<.0001
Corden 2013	3709	−.19 [−0.28, −0.11]	<.0001

not accurately reflect low muscle tissue. We have considered different measures of muscle tissue to all indicate low muscle mass. This is not entirely accurate as differences in muscle cross-sectional area may reflect changes in muscle fibre size as well as fibre atrophy.[44] We have also considered different vascular sites such as brachial-ankle and carotid-femoral PWV as measures of arterial stiffness. However, there is good agreement between these measures and we reasoned that in order to maximise the statistical power of our analyses, it was prudent to consider these measurements together rather than conduct separate brachial-ankle or carotid-femoral PWV analyses. Overall, these differences in study methodology highlight the need for a more standardised approach which would ideally involve measurement of aortic PWV and conducted in healthy individuals.

In conclusion, we identified numerous observational studies that reported on the correlation or association of muscle tissue and arterial stiffness. These results suggest that there was a consistent inverse relationship between the amount of muscle tissue and the level of arterial stiffness across diverse population groups. This finding was consistent across several sensitivity analysis accounting for important risk factors of muscle loss and arterial stiffness. Given the lack of prospective data, determination of whether low muscle mass predicts increases in arterial stiffness over time is warranted. Further, it remains to be seen whether improving muscle mass concurrently improves arterial stiffness. Since, arterial stiffness is predictive of CV mortality and events and hence these events could accelerate the transition of people with low muscle mass to greater disability

and mortality, this systematic review and meta-analysis suggests it may be useful to consider the cardiovascular health of people with low muscle mass.

4 | METHODS

4.1 | Study focus

This study was conducted in accordance with the guidelines outlined in the MOOSE statement (Meta-analysis of Observational Studies in Epidemiology).[45] Studies were eligible if they were observational in design (cross-sectional or longitudinal/prospective), reported a measure of muscle tissue, reported a measure of arterial stiffness and performed a statistical assessment of the relationship between these measures in order to determine the linearity between these measures. Specific exclusion criteria were non-observational studies, animal or cell-based studies, reviews and other meta-analyses and individual case reports. We restricted our analysis to observational studies only as we reasoned that we wanted to be able to establish the natural incidence of both low muscle tissue and increased arterial stiffness. Interventional studies could, depending on the type intervention, potentially alter measures of both arterial stiffness and muscle tissue and thus affect the association between these measures.

4.2 | Literature search

Records were retrieved by searching MEDLINE (archives from 1966-2016) and EMBASE (1946-2016) databases. Records were identified on 23 February 2016 using a search string detailed in Table S1. Titles and abstracts of identified records were screened by a single reviewer (AJR). Reference lists were manually scanned by hand searching. Following title and abstract screening, full-text articles were interrogated to determine eligibility for inclusion. For full-texts unavailable publically, attempts were made to obtain these or specific data directly from the authors to ensure eligibility.

4.3 | Data extraction

Data capture was performed independently by two reviewers (AJR, MNK) with the aid of a data extraction template. The information

captured during data extraction was: the name of the first author, year of publication, study design, sample demographics, measurement of muscle tissue and arterial stiffness, statistical analyses employed and study limitations. These data were then tabulated into a format that enabled comparison of relevant study details. Data relating to the relationship between muscle tissue and arterial stiffness were also tabulated.

4.4 | Quality assessment

Further to data extraction, we performed a quality assessment of studies included in the systematic review. As no standardised quality assessment checklist exists for observational studies reporting on the association between muscle tissue and arterial stiffness, we designed a ten-point semi-quantitative questionnaire using preferred reporting items from MOOSE to judge quality of included studies. Items in our checklist related to study design, generalisability, outcome measurement, statistical analysis and limitations. Items were checked as either "yes", "no" or "unclear" and a score of greater than or equal to 8 (yes responses out of 10) was deemed high quality; scores 5-7 were good quality and scores less than or equal to 4 were poor quality (specific questions are provided in Table S2).

4.5 | Statistical operations

4.5.1 | Meta-analysis eligibility

Studies were included for meta-analysis if they first satisfied the inclusion and exclusion criteria for systematic review and then reported correlation or association statistics (for example Pearson's r correlation coefficient, standardised (β) coefficient, R^2 correlation coefficient or odds ratios) in assessing the relationship between muscle tissue and arterial stiffness. Studies were excluded from meta-analysis, but still kept in the qualitative review, if they reported only descriptive statistics for measures of arterial stiffness according to groups defined on the basis of the amount of muscle tissue (e.g. above or below the median amount of muscle tissue). For studies that reported on multiple sites of muscle tissue (e.g. leg or arm muscle mass) preference for inclusion in meta-analysis dataset was arbitrarily first given to whole body, then appendicular, then leg, then arm, then trunk measures.

4.5.2 | Meta-analysis

Data was first tabulated into a format that enabled visualisation of group data. In order to pool correlation statistics together, we converted r-correlation coefficients into to the Fisher's z scale using the Fisher's Z transformation: $Z = 0.5 \times \ln\left(\frac{1+r}{1-r}\right)$ and calculated the standard error: $SE = \sqrt{\left(\frac{1}{n-3}\right)}$.[46] The Fisher's Z score and its variance are used in the analysis. These data were then synthesised into an inverse-variance model to determine the pooled weighted r correlation and 95% confidence interval. Heterogeneity was determined by the inconsistency statistic (I^2) where a DerSimonian

and Laird random-effects model was applied if I^2 was greater than 50%. A number of sensitivity analyses were performed in order to examine the possible effects of factors that may contribute to muscle loss and arterial stiffness. To assess the effect of age, we divided our analysis into studies with a mean age of ≥60 years and studies with a mean age <60 years and performed the same operations as above. Similarly, though there is good agreement between imaging modalities, to account for potential differences in lean mass measurements we stratified the analysis separating those studies who used DXA and those who used another modality. Finally, whole body muscle mass assessment would best reflect low muscle mass though some studies assessed regional muscle mass such as thigh muscle mass, therefore to investigate the potential effect of imaging site we stratified the analysis into whole body muscle mass assessment and regional muscle mass assessment. Additionally we performed a leave-one-out sensitivity analysis were, sequentially, each study was excluded from the meta-analysis model in order to determine if the summary effect was overly influenced by any single study (which may be the case for a large study group with smaller studies). All statistical operations were performed using RevMan v5.3 software (The Nordic Cochrane Centre, The Cochrane Collaboration 2012) and in consultation with a statistician (MNK).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Supplementary Table 1. Literature search string

Muscle terms [Title/Abstract]		Arterial stiffness terms
muscle		arterial stiffness
muscle mass		vascular stiffness
muscle size		pulse wave velocity
skeletal muscle		pwv
lean mass		augmentation index
appendicular lean mass	AND	pulse pressure
muscle cross sectional area		
skeletal muscle index		
sarcopenia		
muscle loss		

Supplementary Table 1. Summary of quality assessment

Item	Study (32)	(12)	(13)	(14)	(16)	(15)	(17)	(18)	(19)	(20)	(21)	(22)	(23)	(24)	(25)	(26)	(27)	(28)	(29)	(30)	(31)
1	Yes	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
2	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
3	u/c	No	Yes	Yes	Yes	Yes	No	Yes	u/c	No	u/c	No	No	Yes	Yes	No	Yes	No	No	No	Yes
4	Yes	No	Yes	No	Yes	No	Yes	u/c	No	u/c	u/c	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
5	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	No	Yes	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
6	Yes	u/c	Yes	u/c	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
7	Yes	Yes	Yes	Yes	Yes	No	u/c	Yes	u/c	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	u/c	u/c	Yes	Yes
8	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	u/c	u/c	Yes	u/c	Yes	u/c
9	Yes	No	No	Yes	Yes	No	Yes	Yes	No	Yes	Yes	u/c	Yes	Yes	Yes	No	No	Yes	Yes	Yes	Yes
10	Yes	Yes	Yes	Yes	Yes	u/c	Yes	Yes	Yes	Yes	Yes	No	Yes	No	Yes	No	Yes	Yes	Yes	Yes	Yes
Total Yes/10	9	5	8	7	9	4	7	8	4	7	7	5	7	6	9	5	7	7	6	8	8
Quality assessment	High	Good	High	Good	High	Poor	Good	High	Good	Good	Good	Good	Good	Good	High	Good	Good	Good	Good	High	High

Quality criteria: High ≥8; Good = 5-7; Poor ≤4; u/c = unclear

Chapter 2.3: Original Research

Low Relative Lean Mass is Associated with Increased Likelihood of Abdominal Aortic Calcification in Community-Dwelling Older Australians.

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CrossMark

ORIGINAL RESEARCH

Low Relative Lean Mass is Associated with Increased Likelihood of Abdominal Aortic Calcification in Community-Dwelling Older Australians

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Abstract Age-related loss of skeletal muscle is associated with increased risk of functional limitation and cardiovascular (CV) mortality. In the elderly abdominal aortic calcification (AAC) can increase CV risk by altering aortic properties which may raise blood pressure and increase cardiac workload. This study investigated the association between low muscle mass and AAC in community-dwelling older Australians. Data for this cross-sectional analysis were drawn from a 2010 sub-study of the Melbourne Collaborative Cohort Study in the setting of community-dwelling older adults. Three hundred and twenty-seven participants [mean age = 71 ± 6 years; mean BMI = 28 ± 5 kg/m^2; females $n = 199$ (62 %)] had body composition determined by dual-energy x-ray absorptiometry (DXA) and AAC determined by radiography. Participants were stratified into tertiles of sex-specific BMI-normalised appendicular lean mass (ALM). Those in the lowest tertile were considered to have low relative muscle mass. Aortic calcification score (ACS) was determined visually as the extent of calcification on the aortic walls between L1 and L4 vertebrae (range: 0–24). Severe AAC was defined as ACS ≥ 6. Prevalence of any AAC was highest in participants with low relative muscle mass (74 %) compared to the middle (65 %) and upper (53 %) tertiles (p trend = 0.006). The lower ALM/BMI tertile had increased odds (Odds ratio = 2.3; 95 % confidence interval: 1.1–4.6; $p = 0.021$) of having any AAC; and having more severe AAC (2.2; 1.2–4.0; $p = 0.009$) independent of CV risk factors, serum calcium and physical activity. AAC is more prevalent and severe in community-dwelling older adults with low relative muscle mass. Maintaining muscle mass could form part of a broader primary prevention strategy in reducing AAC.

Keywords Sarcopenia · Aortic calcification · Ageing · Cardiovascular disease · Muscle

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Introduction

Average life expectancy for people in developed countries is now between eighty and ninety years and will continue to increase [1]. Older individuals commonly require long-term management for chronic disability and health disorders. Therefore, the ageing population phenomenon imposes a significant health and economic burden on society. Sarcopenia describes the progressive loss of skeletal muscle tissue and function during ageing [2]. The loss of skeletal muscle mass in particular has a number of

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important clinical implications. Recent literature suggests that aside from functional limitations, people with low muscle mass are vulnerable to developing several conditions known to increase cardiovascular (CV) risk including insulin resistance [3] and metabolic syndrome [4].

However, much of the CV disease burden may be related to non-traditional CV risk factors (that is to say, risk factors not normally investigated in routine CV risk monitoring by family physicians) such as vascular stiffness and calcification [5, 6]. Low muscle mass may be related to increased calcification through the indirect effects of muscle loading on bone. The loss of muscle mass may diminish the mechanical loading effect on bone and promote demineralisation as has been demonstrated in individuals who have had sustained bed rest or have spent time in outer space [7]. Previous clinical studies suggest that bone demineralisation is a risk factor for vascular (aortic) calcification and reduced arterial compliance [8]. Low muscle mass may also be associated with arterial calcification through other indirect mechanisms. Sarcopenia has been associated with a chronic low-grade pro-inflammatory milieu, increased oxidative stress and endothelial dysfunction [9]. Inflammatory mediators such as interleukin-6, tumour necrosis factor-α and C-reactive protein can directly affect muscle catabolism and may also act indirectly by reducing insulin sensitivity or lowering growth hormones [10]. These pathways are shared in vascular calcification suggesting a link between low muscle mass and calcification [11].

Despite this evidence, there are few direct investigations [12, 13] of the relationship between low muscle mass and abdominal aortic calcification (AAC), a common site of arterial calcification. Therefore, the aim of this study was to determine if low muscle mass was associated with increased likelihood of aortic calcification in community-dwelling older adults.

Methods

Study Participants

This analysis drew data from a sub-study of participants of the Melbourne Collaborative Cohort Study (MCCS) for which study design and inclusion/exclusion criteria have been described in detail previously [14]. Briefly, the MCCS is a large prospective cohort study of 41,514 (17, 045 men) community-dwelling individuals from Melbourne, Australia aged between 27 and 75 years (of which 99.3 % were aged 40–69 years) at baseline (1990). For the present sub-study, 956 English speakers who had calcium intake <500 mg/day (low) or $\geq$1300 mg/day (high) were approached to complete questionnaires and thoraco-lumbar

radiographs (2010) in order to examine the effects of calcium intake on cardiovascular events [15]. Of the 759 that responded to the invitation, 652 were eligible to participate after excluding those who were using medication(s) known to affect bone and mineral metabolism or had a diagnosis of cancer or other chronic disease known to affect bone and mineral metabolism. Two hundred and forty-five were excluded due to their inability to participate or refused further participation. Finally, 407 participants had imaging performed of which 353 completed whole-body DXA scans and 346 completed radiographs. A total of 327 participants had complete data and were included in this study (Fig. 1). The study protocol was approved by the Cancer Council Victoria's and Melbourne Health's Human Research Ethics Committees.

Clinical and Laboratory Values

For this study, the 327 included participants had a number of assessments performed. Height, weight, waist and hip circumference were obtained as per standard protocols. Briefly, weight was measured in light clothing without shoes using an electronic digital scale to the nearest 0.1 kg (Seca 804, Seca, Germany). Standing height was measured using a stadiometer to the nearest 0.1 cm. Waist circumference was measured to the nearest 0.1 cm using an inelastic plastic fibre measuring tape. The measuring tape was placed around the abdomen at the mid-point between the lowest floating rib and the top of the iliac crest and kept at the level of the umbilicus. Hip circumference was measured in the same standing position with the measuring tape around the maximum circumference of the buttocks. Waist-to-hip ratio (WHR) was calculated by dividing waist circumference by hip circumference. Body mass index (BMI) was calculated as body mass (kg) divided by height (m) squared. Resting blood pressure and heart rate were measured for each participant in a seated position with the cuff at the level of the heart using an automatic blood pressure monitor (Omron Healthcare HEM907, Sydney Australia). Two readings of blood pressure were measured separated by a 5-min interval. Hypertension was defined as a systolic blood pressure >140 mmHg according to the 2011 expert consensus document of the American College of Cardiology and American Heart Association. Physical activity was based on results of the previously validated Community Healthy Activities Model Programme for Seniors (CHAMPS) questionnaire which estimates total weekly caloric expenditure (kcal/week) as a continuous variable [16]. A food frequency questionnaire (FFQ) was used to estimate daily protein intake (g/day) and daily vitamin D intake (μg/day) [17]. Socio-economic status, which may be indicative of healthy living behaviours, was estimated by using the Australian Government socio-

Fig. 1 Participant selection flow diagram

Total participants in MCCS
41, 514

Participants with high/low calcium intake invited to complete questionnaires and radiography/densitometry
956

Responded to invitation
759

Participants excluded due to use of medications known to effect bone and mineral metabolism
107

Satisfied inclusion criteria
652

Participants excluded due to ineligibility or refused further participation
245

Had imaging performed
407
[352 DXA, 346 Radiographs]

Participants excluded due to missing data
25

Participants included
327

economic indexes for areas (SEIFA) Index of Socio-Economic Disadvantage ranking participants on a scale of least to most disadvantaged according to participant postcode at baseline (when they were first enrolled in the MCCS) [18]. Other clinical characteristics relating to history of myocardial infarction (MI), stroke, angina, coronary artery bypass graft (CABG), type 2 diabetes mellitus (T2D), self-reported fractures and smoking status (ever smoked) were obtained by face-to-face interviews and questionnaire. Fasting blood samples were collected by trained phlebotomists. Lipid fractions (total cholesterol [TC], triglycerides [TG], high-density lipoprotein [HDL], low-density lipoprotein [LDL]) serum homocysteine (Hcys) and calcium (Ca) were analysed by technicians at Melbourne Pathology centres. Bone turnover markers, C-terminal peptide (CTX) and pro-collagen type-1 N propeptide (P1NP), were also evaluated as surrogate markers of bone demineralisation. CTX is a marker of bone resorption and P1NP indicates bone formation. Serum vitamin D levels were analysed at the RMIT Drug Discovery Technology laboratories (Melbourne, Australia) using liquid chromatography-tandem mass spectroscopy. Low vitamin D

was defined as serum calcifediol/25(OH)D concentration below 50 nmol/L (=20 ng/mL) according to The Endocrine Society guidelines. Elevated TC was defined as serum TC ≥ 240 mg/dL (≥6.216 mmol/L) according to the U.S. National Institutes of Health guidelines.

Bone Density, Body Composition and AAC Assessment

Bone densitometry and body composition measurements were performed using a Hologic densitometer (QDR 4500 W, Hologic Inc., Bedford, Massachusetts) which uses a fan-beam-based DXA to measure bone mineral density [BMD] (g/cm^2) and bone mineral content (g/cm) and body composition including appendicular lean mass (ALM) and body fat. Standard operating procedures were followed for participant positioning and acquisition [19]. Scans were analysed using Hologic APEX 3.1 software according to standard procedures. There are a number of operational definitions for sarcopenia. Australia does not have a validated low muscle mass/sarcopenia cut-point. The Foundation for the National Institutes of Health (F-NIH) in the

USA recommends assessment of appendicular lean mass normalised to BMI (ALM/BMI) in its definition of sarcopenia [20]. Pooling data from several large cohorts totaling approximately 26,000 individuals, a cut-point of <0.789 for men and <0.512 for women, were established. However, using these cut-points, very few individuals in this study would be considered sarcopenic (male, $n = 22$ (0.06 %); female, $n = 20$ (0.06 %). Therefore, consistent with previous investigations, we defined low muscle mass as the sex-specific lowest tertile for ALM/BMI [4, 21]. Tertiles of ALM/BMI in this study were as follows: (men: lower = <0.867, middle = 0.867–0.943 and upper = >0.943; women: lower = <0.572, middle = 0.572–0.634 and upper = >0.634).

AAC deposits were assessed by way of thoraco-lumbar radiographs, which is a standard imaging modality for AAC detection [22]. AAC was scored using a visually based technique [23] by two trained operators (BK, NK) according to the extent and severity of calcified deposits in the anterior and posterior walls of the abdominal aorta at the level L1–L4 vertebrae [23]. In this technique, for each vertebral segment, calcified deposits were scored 0 for no calcification; 1—one-third or less of the walls were calcified; 2—between one-third and two-thirds of the walls were calcified and 3—more than two-thirds of the walls were calcified. The scores for the anterior and posterior walls were summed meaning each vertebral segment contributes a maximum of score of six to the final score. The final aortic calcification score (ACS) was a composite of all four vertebral segments ranging from a minimum "0" to a maximum of "24" and is thus a non-continuous outcome. If scores between the assessors were inconsistent, a further assessment by both assessors together was performed to achieve agreement. Based on the ACS, participants were stratified in AAC severity groups, defined as "no calcification" (ACS = 0), "moderate calcification" (ACS between 1 and 5), and "severe calcification" (ACS score ≥6).

Statistical Analysis

Continuous and normally distributed variables were reported as mean ± standard deviation (SD); non-parametric or non-continuous variables were presented as median and interquartile range (IQR), and categorical variables were reported as frequency and percentage. Comparison of variables between ALM/BMI tertiles was performed using one-way analysis of variance, Kruskal–Wallis or χ^2 test as appropriate to the exposure variable of interest. The association between clinical variables and the presence of AAC (a binary outcome which we will refer to as AAC_{pres}) was explored by univariable logistic regression. Further, the association between clinical variables and

severity of AAC was explored by ordinal logistic regression where calcification was stratified into groups of increasing ACS. To interpret the ordinal regression results, the odds presented represent the likelihood of being in a higher category of ACS for increases in the exposure (muscle mass loss). In multivariable analysis, we constructed models first adjusted for age and sex and then a fully adjusted model including age and sex, traditional AAC risk factors of smoking, hypertension, stroke, TG, HDL, TC and Hcys as well as variables likely to influence muscle mass and/or calcification including lumbar spine BMD, serum calcium and weekly caloric expenditure. In order to determine the possible influence of obesity in these models, we stratified the cohort into those who were (centrally) obese (defined as a WHR of >0.9 in men and >0.85 in women) and those who were not obese and constructed these models for obese and non-obese groups. As lumbar spine BMD may be contaminated by artefact from vertebral degradation in older age, we constructed this fully adjusted model replacing lumbar spine BMD with femoral neck BMD. Furthermore, as a validation of the decision to use the ALM/BMI definition, we constructed these fully adjusted regression models in tertiles of ALM/h^2 as per the Baumgartner criteria [ALM/h^2 <7.26 in men and <5.45 in women] [24]. Values were considered significant if $p < 0.05$ and all data were analysed using SPSS Statistics v22 (IBM, Armonk, NY, USA).

Results

There were 327 individuals with complete data for analysis; the demographic and clinical characteristics for the included participants are summarised in Table 1. Briefly, the sample predominantly (60 %) female had a mean age of approximately 70 years and the mean BMI was in the overweight range. The individuals with DXA but no radiograph or biochemistry results ($n = 26$) and thus excluded from the final analysis had a mean age of approximately 73 years, the mean BMI was again in the overweight range and there were slightly more females than males (approximately 57 % female).

Of the included sample, those with low relative muscle mass (in the lower tertile of ALM/BMI) had higher body weight, BMI, body fat, higher WHR and more obesity defined by WHR (Table 1) compared to the other tertiles. As expected, this group also had the lowest ALM compared to the other groups. There were no significant differences in lumbar spine or hip BMD between tertiles. There was no significant difference in serum vitamin D level, proportion of individuals who had low vitamin D, daily vitamin D consumption, proportion of individuals who were considered to be disadvantaged, total daily

Table 1 Descriptive statistics of participant demographic and clinical characteristics

Variable n	Tertile of ALM/BMI				
	All 327	Lower 108	Middle 110	Upper 109	p
Age (years)	70.5 ± 5.5	70.9 ± 5.6	71.2 ± 5.6	69.0 ± 4.9	0.001
Age > 80 [n (%)]	19 (5.8)	5 (4.6)	11 (10.0)	3 (2.8)	0.067
Male sex [n (%)]	128 (39.1)	42 (38.9)	43 (39.1)	43 (39.4)	0.996
Weight (kg)	75.9 ± 15.3	80.0 ± 16.2	74.1 ± 13.0	72.8 ± 14.2	0.002
Height (kg)	1.6 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	<0.001
BMI (kg/m^2)	28.1 ± 5.2	31.2 ± 5.4	27.3 ± 3.4	25.5 ± 3.7	0.001
WHR (unitless)	0.82 ± 0.10	0.83 ± 0.12	0.82 ± 0.09	0.80 ± 0.09	0.037
WHR (#obese [%])	91 (27.8)	36 (33.3)	33 (30)	22 (20.2)	0.080
WC (cm)	82.8 ± 13.5	85.4 ± 15.2	82.5 ± 11.9	80.6 ± 13.0	0.006
ALM (kg)	20.1 ± 5.2	19.3 ± 4.6	19.6 ± 4.7	21.1 ± 5.7	0.050
Body fat (%)	35.8 ± 8.1	39.9 ± 7.3	35.2 ± 7.4	32.3 ± 6.9	<0.001
Body fat (kg)	26.1 ± 9.4	26.6 ± 7.8	26.8 ± 10.2	25.1 ± 9.3	0.178
MI [n (%)]	18 (5.5)	9 (8.3)	5 (4.5)	4 (3.7)	0.278
CABG [n (%)]	11 (3.4)	4 (3.7)	2 (1.8)	5 (4.6)	0.510
Angina [n (%)]	16 (5.0)	9 (8.5)	2 (1.8)	5 (4.6)	0.078
Stroke [n (%)]	21 (6.5)	7 (6.6)	9 (8.3)	5 (4.7)	0.566
Hypertension [n (%)]	100 (30.6)	48 (44.4)	29 (26.4)	23 (21.1)	<0.001
Fracture [n (%)]	94 (28.8)	32 (29.9)	25 (22.7)	37 (33.9)	0.178
T2D [n (%)]	31 (9.7)	11 (10.7)	13 (11.8)	7 (6.5)	0.387
Smoker [n (%)]	107 (32.8)	29 (27.1)	37 (33.6)	41 (37.6)	0.252
ACS (median)	3 (0-17)	4 (0–17)	3 (0–16)	1 (0–14)	0.001
AAC [n (%)]	209 (63.9)	80 (74.1)	71 (64.5)	58 (53.2)	0.006
Severe ACS [n (%)]	101 (31.6)	40 (38.1)	38 (35.2)	23 (21.5)	0.009
Most disadvantaged [n (%)]	77 (14)	18 (16.7)	28 (25.5)	30 (27.5)	0.196
Least disadvantaged [n (%)]	49 (8.9)	17 (15.7)	15 (13.6)	13 (11.9)	0.766
Caloric expenditure (kcal/week)	3.73 ± 2.98	3.49 ± 3.15	3.87 ± 3.29	4.22 ± 3.02	0.067
Protein intake (g/day)	90.2 ± 13.2	92.8 ± 31.1	88.3 ± 30.8	88.6 ± 32.0	0.419
Vitamin D (ng/mL)[a]	74.8 ± 26.4	73.3 ± 28.8	77.4 ± 24.1	74.3 ± 27.6	0.696
Vitamin D <50 ng/mL (n[%])	63 (20.3)	21 (20.6)	19 (17.8)	23 (22.5)	0.686
Vitamin D intake (μg/day)	3.2 ± 2.2	3.3 ± 2.0	3.5 ± 2.9	3.0 ± 1.7	0.692
Lumbar BMD (g/cm^2)	1.123 ± 0.209	1.115 ± 0.191	1.120 ± 0.203	1.113 ± 0.215	0.967
Hip BMD (g/cm^2)	0.922 ± 0.145	0.940 ± 0.139	0.911 ± 0.138	0.905 ± 0.145	0.127
Femoral neck (g/cm^2)	0.713 ± 0.191	0.694 ± 0.217	0.724 ± 0.176	0.720 ± 0.176	0.694
TC (mmol/L)	5.0 ± 1.5	5.1 ± 1.1	5.3 ± 1.1	5.3 ± 1.1	0.090
TG (mmol/L)	1.3 ± 0.6	1.5 ± 0.7	1.3 ± 0.6	1.2 ± 0.5	0.011
HDL (mmol/L)	1.6 ± 0.5	1.5 ± 0.5	1.6 ± 0.5	1.7 ± 0.5	0.366
LDL (mmol/L)	3.0 ± 1.0	2.9 ± 1.1	3.1 ± 1.0	3.0 ± 0.9	0.185
High TC [n (%)]	131 (40.8)	48 (45.7)	45 (41.7)	38 (35.2)	0.288
Hcys (mmol/L)	10.2 ± 2.6	10.6 ± 2.8	10.0 ± 2.0	9.6 ± 2.7	0.018
Ca (μmol/L)[a]	2.4 ± 0.1	2.3 ± 0.1	2.4 ± 0.1	2.3 ± 0.1	0.371
Ca intake ≥ 1300 mg/day [n (%)]	165 (50.5)	50 (46.3)	49 (44.5)	66 (60.6)	0.035
CTX (pg/mL)	419.6 ± 183.6	371 ± 166	407 ± 173	456 ± 187	0.001
P1NP (ng/mL)	42.7 ± 19.1	39.3 ± 15.6	39.6 ± 16.5	45.3 ± 20.4	0.230

[a] ANOVA performed as data was normally distributed

protein intake, lumbar spine, femoral neck or total hip BMD, serum Ca, P1NP or number of previous fractures between the tertiles of ALM/BMI. However, the low relative muscle mass group had the lowest proportion of high daily calcium intake and lowest mean serum CTX, more hypertensives, and greater mean TG and Hcys. There were no differences in other clinical features including caloric expenditure, MI, stroke, smoking, angina, CABG, T2D and blood lipids between the tertiles. ACS was highest in the lowest tertile compared to the middle and upper tertiles (Table 1). Similarly, AAC_{pres} and severe AAC were significantly more prevalent in the group with low relative muscle mass.

In multivariable analyses, there was an inverse association between relative muscle mass and calcification in the aorta. The lowest tertile of ALM/BMI was significantly associated with an approximately two-and-a-half-fold higher likelihood of having any AAC_{pres} and having severe AAC relative to the upper tertile in an unadjusted model (Model 1). These associations were attenuated slightly but remained significant after adjustment for age and sex (Model 2). Further adjustment for traditional CV risk factors as well as serum calcium and physical activity (Model 3) resulted in slightly increased likelihood of having any AAC_{pres} and having severe AAC relative to the upper tertile (Table 2). In addition to ALM/BMI, the following confounders were significantly associated with AAC_{pres} in the fully adjusted model: age [in years] (Odds = 1.09, $p = 0.001$); hypertension [systolic blood pressure >140 mmHg] (Odds = 2.09, $p = 0.007$) and smoking [ever smoked—yes vs. never smoked] (3.12, $p < 0.001$). In

order to determine the possible influence of obesity in these models, as those in the lower ALM/BMI tertile had greater adiposity, we stratified the cohort into those who were centrally obese (defined as a WHR of >0.9 in men and >0.85 in women) and those who were not obese. The same models were constructed as per Table 2 and in obese individuals, in the fully adjusted model ALM/BMI was not associated with increased likelihood of AAC_{pres} (Table 3). However, in non-obese individuals, the lower ALM/BMI tertile was associated with an approximately three-fold increased likelihood of having any AAC_{pres} and an approximately two-fold increased likelihood of having severe AAC (Table 4.). As lumbar spine BMD may be contaminated by artefact from vertebral degradation, we constructed a fully adjusted model using femoral neck BMD as a cofounder. A similar pattern of results emerged; the lower tertile of ALM/BMI was associated with a near twofold increased likelihood for the presence of AAC_{pres} but this was not significant (Odds = 1.88, $p = 0.082$). However, lower tertile of ALM/BMI was significantly associated with increased likelihood of having severe AAC (Odds 1.86, $p = 0.044$) (Table 5). When this analysis was separated into obese and non-obese defined by WHR, ALM/BMI not associated with increased presence or severity of AAC. In this analysis, there was a mixed pattern of results. Non-obese individuals had a higher likelihood (non-significant) of AAC_{pres} compared to obese individuals in the lower tertile of ALM/BMI; however, obese individuals had higher odds (non-significant) of severe AAC compared to non-obese individuals in the lower tertile of ALM/BMI (Table 5).

Table 2 Logistic regression models for the outcome of AAC (whole sample)

Model	ALM/BMI	Presence			Severity (ordinal)		
		Odds	95 %CI	p	Odds	95 %CI	p
Model 1	Upper	Ref	Ref	Ref	Ref	Ref	Ref
	Middle	1.60	0.93–2.75	0.09	**1.79**	**1.08–2.96**	**0.02**
	Lower	**2.51**	**1.42–4.45**	**0.002**	2.417	1.456–3.97	0.001
Model 2	Upper	Ref	Ref	Ref	Ref	Ref	Ref
	Middle	1.36	0.77–2.38	0.297	1.54	0.92–2.57	0.10
	Lower	**2.15**	**1.19–3.88**	**0.01**	**2.13**	**1.28–3.56**	**0.004**
Model 3	Upper	Ref	Ref	Ref	Ref	Ref	Ref
	Middle	1.59	0.837–3.02	0.16	1.72	0.97–3.06	0.064
	Lower	**2.56**	**1.29–5.11**	**0.007**	**2.38**	**1.32–4.29**	**0.004**

Model 1—unadjusted; Model 2—age and sex; Model 3—age, sex, smoking, hypertension, stroke, TG, HDL, TC, Ca, Hcys, Lumbar BMD and caloric expenditure. *Ref* Reference. n.b. The odds presented in the severity column represent the likelihood of having a higher ACS score; in other words every unit increase in muscle mass loss will be associated with an approximately two-and-a-half-fold likelihood increase in being in a higher ACS category

Bold values correspond to significant results ($p < 0.05$)

Table 3 Logistic regression models for the outcome of AAC in obese individuals

Model	ALM/BMI	Presence			Severity		
		Odds	95 %CI	*p*	Odds	95 %CI	*p*
Model 1	Upper	Ref	Ref	Ref	Ref	Ref	Ref
	Middle	0.88	0.24–3.62	0.85	1.49	0.49–4.51	0.48
	Lower	1.83	0.44–7.62	0.41	2.29	0.75–6.96	0.14
Model 2	Upper	Ref	Ref	Ref	Ref	Ref	Ref
	Middle	0.69	0.18–2.72	0.60	1.05	0.33–3.35	0.93
	Lower	1.85	0.42–8.14	0.42	2.31	0.73–7.28	0.15
Model 3	Upper	Ref	Ref	Ref	Ref	Ref	Ref
	Middle	0.41	0.08–1.97	0.26	0.57	0.16–2.03	0.38
	Lower	2.34	0.41–13.49	0.34	2.33	0.65–8.44	0.19

Model 1—unadjusted; Model 2—age and sex; Model 3—age, sex, smoking, hypertension, stroke, TG, HDL, TC, Ca, Hcys, Lumbar BMD and caloric expenditure. *Ref* Reference

Table 4 Logistic regression models for the outcome of AAC in non-obese individuals

Model	ALM/BMI	Presence			Severity		
		Odds	95 %CI	p	Odds	95 %CI	*p*
Model 1	Upper	Ref	Ref	Ref	Ref	Ref	Ref
	Middle	**2.24**	**1.11–4.50**	**0.024**	**2.03**	**1.06–3.89**	**0.033**
	Lower	**3.78**	**1.81–7.90**	**<0.001**	**3.06**	**1.60–5.86**	**0.001**
Model 2	Upper	Ref	Ref	Ref	Ref	Ref	Ref
	Middle	2.02	0.98–4.14	0.056	1.86	0.97-3.63	0.062
	Lower	**3.32**	**1.56–7.06**	**0.002**	**2.68**	**1.38–5.19**	**0.003**
Model 3	Upper	Ref	Ref	Ref	Ref	Ref	Ref
	Middle	2.00	0.95–4.21	0.065	1.87	0.95–3.68	0.069
	Lower	**2.83**	**1.27–6.31**	**0.011**	**2.34**	**1.16–4.74**	**0.017**

Model 1—unadjusted; Model 2—age and sex; Model 3—age, sex, smoking, hypertension, stroke, TG, HDL, TC, Ca, Hcys, Lumbar BMD and caloric expenditure. *Ref* Reference

Bold values correspond to significant results ($p < 0.05$)

Table 5 Fully adjusted logistic regression models for the outcome of AAC including femoral neck BMD (instead of lumbar spine) in the model

Sample	ALM/BMI	Presence			Severity		
		Odds	95 %CI	*p*	Odds	95 %CI	*p*
Whole	Upper	Ref	Ref	Ref	Ref	Ref	Ref
	Middle	1.09	0.53–2.21	0.812	1.48	0.82-2.66	0.184
	Lower	1.88	0.92–3.83	0.082	**1.86**	**1.01–3.41**	**0.044**
Obese	Upper	Ref	Ref	Ref	Ref	Ref	Ref
	Middle	0.40	0.07–2.05	0.274	0.852	0.25–2.88	0.797
	Lower	1.28	0.21–7.84	0.785	2.12	0.51–8.70	0.297
Non-Obese	Upper	Ref	Ref	Ref	Ref	Ref	Ref
	Middle	1.34	0.57–3.11	0.49	1.65	0.81–3.38	0.165
	Lower	2.20	0.95–5.06	0.063	1.72	0.85–3.48	0.130

Bold values correspond to significant results ($p < 0.05$)

Discussion

This cross-sectional study of community-dwelling older Australians is the first to our knowledge to demonstrate that those low relative muscle mass is independently associated with over two-fold higher likelihood of any and severe levels of aortic calcification. Maintenance of muscle mass during ageing may well be beneficial with regard to calcification and as such, primary prevention strategies should incorporate some means to increase muscle mass as well as address other important risk factors in reducing risk for cardiovascular disease.

One of the criticisms of ALM/BMI index for sarcopenia is that it is a proxy measure of obesity, as reflected by the fact that those in the lowest tertile of ALM/BMI had greater adiposity. This highlights the need for a sarcopenia definition to incorporate a measure of muscle function such as gait speed or grip strength [25]. After stratifying by central obesity status, the relationship between low relative muscle mass and AAC$_{pres}$ was significant only in non-obese participants. This may be related to the fact that general adiposity is not a significant risk factor for vascular calcification while regional adiposity, such as visceral adipose stores, may be related [26]. Further, given that low muscle mass has already been demonstrated to have prognostic value in other CV diseases such as critical limb ischemia [27], older adults with low muscle mass should be considered for CV risk assessment.

Our results are consistent with previous studies investigating surrogate markers of calcification (e.g., increased pulse wave velocity and carotid-intima media thickness). The present study advances on previous literature by demonstrating a direct relationship between whole-body muscle mass and aortic calcification (providing better CV risk prediction compared to surrogates) [28]. Nevertheless, the method of muscle mass assessment may influence this association given abdominal muscle is not associated with calcified atherosclerosis [12] and ALM (adjusted for height) is not associated with pulse pressure [29].

Low muscle mass may contribute to increased calcification through mechanisms including insulin resistance, bone demineralisation, inflammation, oxidative stress and endothelial dysfunction. Skeletal muscle is the body's largest glucose utilising organ. Severe muscle loss can impair one's glucose disposal capacity leading to impaired glucose tolerance and subsequent insulin resistance [30]. Insulin resistance has previously been linked to vascular calcification through oxidative stress damaging the vascular endothelium [31, 32]. Inflammation may also arise from the presence of non-contractile, non-functional adipose tissue, [inter-/intra-muscular adipose tissue (IMAT)] which gradually replaces lost muscle tissue during ageing. In human obesity, IMAT has been associated with inflammation and muscle triglyceride burden was associated with serum IL-17 levels and the development of early atherosclerosis [33, 34]. Finally, low muscle mass may contribute to increased calcification through bone mechanisms. Bone cells are responsive to mechanical loading. Low muscle mass decreases loading on bone which may promote bone catabolism and through cellular mechanisms lead to vascular calcification [35, 36].

Serum calcium has been associated with an increased risk of myocardial infarction (thought to be the result of calcified coronary arteries) [37]. Participants for this study were invited from the MCCS based on either low (<500 mg/day) or high (≥1300 mg/day) dietary calcium intake. There were more individuals with high dietary calcium intake in the upper tertile of ALM/BMI, which had the lowest median ACS. This supports data from the Framingham Heart Study which prospectively showed calcium intake did not increase coronary artery calcification in older people [38]. Low relative muscle mass in the present study however was not associated with increased odds for calcification after adjustment for calcium intake. This may be related to residual confounding because a higher dietary calcium intake may reflect a generally healthier lifestyle and thus reduce the risk of aortic disease as evidenced by a prospective study in older adults demonstrating high dietary calcium being associated with reduced mortality and CV risk [15]. Similarly, self-reported PA was not associated with calcification although a trend for participants with the highest median ACS to have the lowest amount of PA was observed. It is possible that self-reported PA is insufficiently sensitive to detect differences in physical activity between levels of AAC$_{pres}$, given that previous longitudinal studies have reported that maintaining PA is associated with reduced arterial stiffness and reduced incidence and progression of vascular calcification [39]. Certain modalities and intensities of PA, as opposed to overall energy expenditure, may have a stronger influence on AAC$_{pres}$. Indeed an interventional study demonstrated that aerobic exercise may reduce endothelial dysfunction [40], whereas load bearing exercises, effective at increasing muscle mass, may have adverse effects on arterial stiffness [41].

This study is limited by a cross-sectional design and its findings do not imply causation. Second, these results are relevant to low muscle mass only and associations may differ with functional components of sarcopenia. Third, we did not use previously published low muscle mass cut-points. Using the Baumgartner criteria [24], less than 10 % of the sample had low muscle mass and there was no association with calcification. However, in the whole sample the lower tertile of ALM/h^2 had increased likelihood of severe calcification (odds ratio = 1.89, p = 0.040)

(Online Resources 1, 2). Height declines during ageing and thus lean mass appears to increase using the Baumgartner criteria. Thus, the FNIH ALM/BMI criteria [20] may be a better predictor of aortic calcification than ALM/h^2. Further, in this study, there were no differences in protein intake or socio-economic status across ALM tertiles suggesting other factors may contribute to low relative muscle mass. Australians tend to have low prevalence of low muscle mass using cut-points derived from international populations [42]. We attempted to overcome this limitation by defining low relative muscle mass as the lowest tertile of ALM/BMI. In order to strengthen our definition of sarcopenia it would be ideal to include a measure of muscle function such as hand grip strength or gait speed as a recent evaluation of a number of operational definitions determined that those including measures of muscle function had the highest predictive value for mortality [25]. Fourth, these results are only generalisable to relatively healthy community-dwelling older adults. Fifth, degenerative changes in the lumbar spine may produce artefacts on DXA when assessing lumbar spine BMD. We thus produced fully adjusted regression models including femoral neck BMD instead of lumbar spine and found that low relative muscle mass was only associated with the severity but not presence of calcification (Table 5). Finally, patients were recruited on the basis of having low (<500 mg/day) or high (≥1300 mg/day) calcium intake and thus there may be some selection bias. However, they have reported there is currently little evidence that high dietary calcium intake increases vascular calcification [43].

In conclusion, in community-dwelling older Australian adults, low relative muscle mass was associated with an approximate two-times higher likelihood of having any or severe aortic calcification. Prospective studies are required to determine whether age-related declines in skeletal muscle mass are an independent predictor of vascular calcification in older adults, nevertheless maintaining muscle mass could form part of a broader primary prevention strategy in reducing AAC.

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Supplementary Data

Online Resource 1. Sample demographics based on Baumgartner sarcopenia thresholds

Variable	Sarcopenia	No Sarcopenia	p
n	32	295	
Age (years)	71.7±5.9	70.4±5.4	0.256
Male sex [n(%)]	13 [40.6]	115 [39.0]	0.851
BMI (kg/m^2)	**22.6±2.3**	**28.7±4.9**	**<0.001**
WHR (#obese [%])	0.76±0.07	0.82±0.10	0.053
AAC [n(%)]	20 [62.5]	189 [64.1]	0.849
ACS (median)	3 [0-16]	2.5 [0-17]	0.807

Online Resource 2. Fully adjusted logistic regression models for the outcome of AAC using Baumgartner (ALM/h^2) thresholds for low relative muscle mass

Sample	ALM/h^2	Presence			Severity		
		Odds	95%CI	p	Odds	95%CI	p
Whole	Upper	Ref	Ref	Ref	Ref	Ref	Ref
	Middle	1.08	0.53-2.19	0.832	1.50	0.83-2.69	0.171
	Lower	1.84	0.90-3.75	0.093	**1.89**	**1.03-3.48**	**0.040**
Obese	Upper	Ref	Ref	Ref	Ref	Ref	Ref
	Middle	0.42	0.08-2.09	0.295	0.85	0.25-2.82	0.793
	Lower	1.30	0.22-7.68	0.771	2.22	0.55-9.02	0.262
Non-Obese	Upper	Ref	Ref	Ref	Ref	Ref	Ref
	Middle	1.32	0.56-3.08	0.514	1.69	0.82-3.47	0.149
	Lower	2.16	0.93-4.96	0.070	1.77	0.87-3.61	0.114

Chapter 2.4: Original Research

Aortic Calcification is Associated with Five-Year Decline in Handgrip Strength in Older Women

Rodríguez AJ, Lewis JR, Kiel DP, Schousboe JT, Scott D, Ebeling PR, Prince RL

ORIGINAL RESEARCH

CrossMark

Aortic Calcification is Associated with Five-Year Decline in Handgrip Strength in Older Women

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Abstract

The objective of the study was to determine the association between AAC and neuromuscular function over 5 years. Participants in this study were ambulant women over 70 years old residing in Perth, Western Australia who participated in the Calcium Intake Fracture Outcomes Study, a randomised controlled trial of calcium supplementation. 1046 women (mean age $= 74.9 \pm 2.6$ years; BMI $= 27.1 \pm 4.4$ kg/m^2) were included. Lateral spine images captured during bone density testing were scored for AAC (AAC24; 0–24) at baseline. Severe AAC (AAC$_{sev}$) was defined using established cut points (AAC24 ≥ 6). At baseline and follow-up, isometric grip strength was assessed using a dynamometer. Mobility was assessed by the Timed-Up-and-Go (TUG) test. Using pre-defined criteria, muscle weakness was considered as grip strength < 22 kg and poor mobility defined as TUG > 10.2 s. A subset of women had appendicular lean mass (ALM) determined by dual-energy X-ray absorptiometry at baseline and follow-up ($n = 261$). AAC$_{sev}$ was evident in 193 (18.5%) women. Average decline in grip strength after 5 years was greater in those with AAC$_{sev}$ than those without (3.6 ± 3.7 vs. 2.9 ± 4.2 kg; $p = 0.034$). This remained significant after adjustment for age, treatment allocation, diabetes, smoking history, renal function, medical record-derived prevalent vascular disease, BMI and physical activity ($\beta = -0.184$; 95% confidence interval: $-0.361, -0.008$; $p = 0.040$). AAC$_{sev}$ was not associated with 5-year changes in TUG or ALM in univariable or multivariable analyses (all $p > 0.05$). In older women, severe aortic calcification was associated with greater 5-year decline in muscle strength, but not TUG or ALM. These findings support the concept that vascular disease may have an effect on the loss of muscular strength.

Keywords Aortic calcification · Physical function · Grip strength · Mobility · Older women

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Introduction

With advancing age, there is a progressive loss of muscle mass and function which promotes the onset of disability and increases the risk of falls and fractures [1]. Numerous factors are thought to promote this functional decline, including progressive worsening of co-morbidities such as diabetes and heart disease as well as lifestyle factors such as poor nutrient intake, smoking and reduced amounts of physical activity [2]. One area that has not been adequately described is the effects of vascular disease on the neuromuscular system.

Atherosclerotic vascular diseases also contribute to the development of functional decline. Calcification in the aorta is indicative of generalised atherosclerosis at other vascular beds and coronary artery calcification [3]. It is also associated with future cardiovascular events and deaths [4, 5]. Early abdominal aortic atherosclerotic lesions consisting of lipid-laden macrophages and vascular smooth muscle cells are common in adolescents and can either disappear or progress further to become more advanced atherosclerotic lesions [6]. Calcified abdominal aortic lesions are evidence of advanced atherosclerotic plaques that are easily visualised as raised areas by non-invasive imaging modalities [7]. Additionally, abdominal aortic calcification (AAC) may arise as a consequence of medial arteriosclerosis, or both processes can occur concurrently [8].

Epidemiological studies have reported that vascular calcification and musculoskeletal function are both predictive of fractures. Additionally, vascular calcification and musculoskeletal function may share common biological pathways [9]. The composition of calcified atherosclerotic lesions shares some features of bone. Similarly, the biological processes involved in vascular calcification share a number of features with age-related musculoskeletal decline such as decreased endothelial expression of the vitamin D receptor and downregulation of sclerostin in tissue [10, 11].

Recent studies implicate vascular disease as a contributor to age-related changes in muscle. A previous cross-sectional study of healthy older men and women demonstrated that the presence and severity of AAC was associated with low muscle mass [12]. Functional decline likely precedes and may even exceed rates of loss of muscle mass during ageing [13], but the association between AAC and physical function and decline is unknown. Previous observational studies of advanced atherosclerosis (such as carotid intima-media thickness and arterial stiffness) have shown that these surrogates of advanced atherosclerosis are higher in individuals with low handgrip strength [14] and that walking speeds and mobility decline

with the presence of arterial plaques [15, 16]. However, it is unclear if AAC is a cause or consequence of functional decline based on these observations. We therefore sought to determine whether AAC measured at a single time point was associated with functional decline in a population of community-dwelling older women over 5 years.

Methods

This study was a secondary analysis of the Calcium Intake Fracture Outcome Study (CAIFOS) [17]. Participants in this study were originally recruited in 1998 for a 5-year, double-blind, randomised controlled trial (RCT) of daily calcium supplementation to prevent osteoporotic fracture, whose recruitment strategy has been detailed elsewhere [17]. Briefly, women aged 70 years and older were recruited from the general population in Western Australia. Of the 5586 women approached, 1500 were recruited into the study. All participants were ambulant, did not receive medication or have co-morbidities that could affect bone metabolism. Participants received 1.2 g of calcium carbonate daily or a matching placebo.

For the present study, participants who had AAC24 scores assessed from lateral spine dual-energy X-ray absorptiometry (DXA) scans at entry to the study at baseline in 1998 ($n = 207$) or 1999 ($n = 839$), and muscle strength and mobility assessed at baseline and the 5 year follow-up from their initial study visit in 2003, were included (total $n = 1046$) (Fig. 1). All participants completed anthropometry and a validated food frequency questionnaire [18]. Creatinine was measured using an isotope dilution mass spectrometry (IDMS) traceable Jaffe kinetic assay on a Hitachi 917 analyzer (Roche Diagnostics GmbH, Mannheim Germany). Estimated glomerular filtration rate (eGFR) using creatinine and cystatin C was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) [19]. Impaired renal function was defined as an eGFR < 60 mL/min/1.73 m^2. Prevalent atherosclerotic vascular disease (ASVD) was determined by atherosclerotic hospitalisations which were retrieved from the Western Australian Data Linkage System (WADLS) for each of the study participants. Prevalent atherosclerotic disease were defined using the principal Hospital discharge diagnosis codes with a look-back period of approximately 18 years depending on the date of clinic visit (1980 to date of clinical visit) from the International Classification of Diseases, Injuries and Causes of Death Clinical Modification (ICD-9-CM) [21]. These codes included ischemic heart disease (ICD-9-CM codes 410–414); heart failure (ICD-9-CM code 428); cerebrovascular disease excluding haemorrhage (ICD-9-CM codes 433–438); and peripheral arterial disease (ICD-9-CM codes 440–444). Diabetes status was determined by the medical

Fig. 1 Overview of the study design
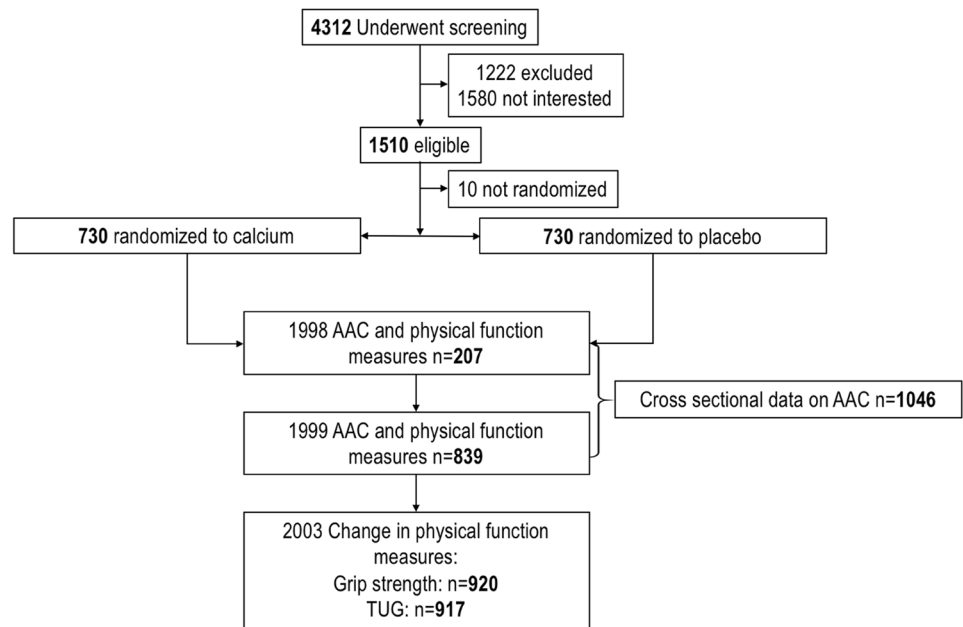
history of study participants and verified by their primary care provider where possible. These data were coded using the International Classification of Primary Care—Plus (ICPC-Plus) method [20]. The coding methodology allows aggregation of different terms for similar pathologic entities as defined by the International Classification of Diseases and related Health Problems, 10th Revision (ICD-10) coding system. These data were then used to determine the presence of pre-existing diabetes (T89001-90009).

Hand grip strength was determined using a Jamar hand dynamometer (Lafayette Instrument Company, USA). Three repeated measures were taken for each hand and the highest recorded strength in the dominant hand was considered the maximum grip strength. Muscle weakness was defined as a maximum grip strength < 22 kg, as this threshold identified clinically relevant weakness in older women [21]. Mobility was assessed by the timed up and go (TUG) test, in which a participant was timed while rising from a chair, walking 3 m, turning and returning to sit on the chair. A TUG time > 10.2 s was considered to be slow, and was referred to as "mobility impairment". This threshold was previously shown to be predictive of fractures in older women [1]. Physical activity was assessed by a demographic questionnaire where participants reported their type of activity and hours per week to the question "Please list any sports recreation or regular physical activity, including walking, that you undertook in the last 3 months" [22]. The energy cost of such activities is given in METs (1 MET accounts for an individual's basal metabolic rate and equals ~1 kcal/kg/h). Activity levels (kcal/day) were calculated by multiplying frequency, duration, energy cost of the activities and the body weight of individuals [23]. Total cholesterol concentrations were determined using a Hitachi 917 auto

analyser (Roche diagnostics). Plasma $25OHD_2$ and $25OHD_3$ concentrations were determined using a validated LC-MS/MS (Liquid Chromatography Tandem Mass Spectrometry) method at the RDDT Laboratories (Bundoora, VIC, Australia) according to published methodology and summed to obtained total plasma 25OHD concentration for each individual [24]. Between-run coefficients of variation (CVs) were 10.1% at a $25OHD_2$ mean concentration of 12 nmol/L and 11.3% at a $25OHD_3$ mean concentration of 60 nmol/L. These were categorised into normal (≥ 50 nmol/L), insufficient (<50 nmol/L) and deficient (<25 nmol/L). All AAC scores from 0 to 24 were derived from digitally enhanced lateral single-energy images of the thoraco-lumbar spine using a Hologic 4500A bone densitometer (Hologic, Bedford, MA, USA). A single experienced investigator (JTS) read all images using the validated semi quantitative scoring system [25]. The use of DXA to detect AAC has been validated against radiography where inter-class correlation coefficient between two readers on DXA was high (0.89: 0.80–0.94) and comparable to radiography (0.92; 0.88–0.95) [26]. Two other studies have shown similar agreement between methods [27, 28]. The AAC24 point system scores aortic calcification relative to each vertebral height (L1–L4) and is scored as 0 (no calcification), 1 (< one-third of the aortic wall), 2 (> one-third to two-thirds of the aortic wall) or 3 (> two-thirds of the aortic wall) for both the anterior and posterior aortic walls giving a maximum possible score of up to 24. Severity of AAC was also categorised using previously published groupings: low (AAC24 score 0 or 1); moderate (AAC24 score 2–5); and severe (AAC24 score > 5). AAC was categorised as not severe (AAC24 score ≤ 5) or severe (AAC24 score > 5). In those women who had AAC measured, 261 had appendicular lean mass (ALM) determined by whole-body

dual-energy X-ray absorptiometry (DXA) (Hologic, Bedford, MA, USA) at baseline and again at 5 years [29].

Statistical Analyses

Normally distributed variables were reported as mean ± standard deviation (SD), non-normally distributed variables were presented as median and interquartile range (IQR), while categorical variables were reported as frequency and percentage. Comparison of demographic, physical and clinical variables stratified by AAC severity was performed using t test, Mann–Whitney U test, χ^2 test or Mantel–Haenszel Chi-squared test for trend as appropriate to data distribution. Given that the aortic calcification score is not normally distributed, Spearman's rank correlation and age-adjusted partial correlation statistics were calculated for the correlation between calcification scores and functional measures, energy expenditure and anthropometric measures. Logistic regression models were constructed to determine if severe AAC was cross-sectionally associated with muscle weakness or mobility impairment at baseline. Linear regression models were constructed to determine if severe AAC was cross-sectionally associated with handgrip strength or TUG at baseline. Further, linear regression models were constructed to determine if severe AAC was associated with 5-year changes in handgrip strength or TUG. All regression models were constructed first as a minimally adjusted model controlling for age and treatment allocation and subsequently adjusted for body mass index (BMI) and relevant risk factors which included diabetes status, smoking status, ASVD, renal function and metabolic equivalents. As only a subset of women had ALM both at baseline and follow-up ($n = 261$), these analyses were repeated in women with these measures stratified by skeletal muscle mass to determine if changes in physical function may be attributable to underlying conditions already affecting muscle mass. Low muscle mass was considered to be an ALM relative to BMI less than 0.512 in line with the Foundation for National Institute of Health (FNIH) definition of sarcopenia [30]. Regression coefficients are presented as the standard deviation increases/decreases in outcome associated with having severe AAC. All data were analysed using STATA v14.2 [StataCorp, 4905 Lakeway Dr College Station, TX, USA]. Values were considered statistically significant if $p < 0.05$ and if the 95% confidence interval (95% CI) did not cross unity.

Results

Baseline data are shown in Table 1. Evidence of any calcification was seen in 762 (73%) of individuals and severe calcification (AAC24 score > 5) was evident in 193 (18%) individuals. Grip strength weakness, defined as maximum grip strength < 22 kg, was evident in 629 women (60.1%) and 32.6% had mobility impairment ($n = 341$) defined as a TUG time > 10.2 s. Of those women with severe AAC, the frequency of allocation to calcium treatment was 45.6% ($n = 88/193$) and this did not differ significantly ($p = 0.331$) from those women with not severe AAC (49.4%; $n = 422/853$). There were no differences in total cholesterol or vitamin D status amongst women with and without severe AAC.

Individuals with severe AAC were significantly older, had lower mean BMI, upper arm girth, triceps skin folds, and were more likely to be smokers, have ASVD, and use statins, low-dose aspirin, and any cardiovascular medication. Neither grip strength nor TUG time at baseline differed between those with and without severe AAC (Table 1). There were no significant correlations for AAC24 scores with baseline handgrip strength, TUG or ALM. AAC24 was negatively but weakly correlated with baseline BMI in an age-adjusted partial correlation model ($r = -0.067$; $p = 0.030$) (Table 2).

One hundred and twenty-six women did not have grip strength recorded and 130 women did not have TUG recorded at follow-up. These women were slightly older (75.3 ± 2.8 vs. 74.8 ± 2.5 years; $p = 0.052$) but were not heavier (BMI = 27.2 ± 5.1 vs. 27.1 ± 4.3 kg/m^2; $p = 0.792$). In addition, women lost to follow-up had a lower baseline grip strength (19.7 ± 4.8 vs. 20.8 ± 4.7; $p = 0.019$) and a slower baseline TUG (10.4 ± 2.9 vs. 9.5 ± 2.6; $p = 0.005$). In women with follow-up functional measures, the median reduction in grip strength over 5 years was 3.0 kg (interquartile range: −5.5 to −0.75 kg; $n = 920$). The average decline in grip strength in individuals with severe AAC (3.6 ± 3.7 kg) was greater than in those without (2.9 ± 4.2 kg) ($p = 0.034$). The median increase in TUG times was 1.26 s (IQR: −0.02, 2.64; $n = 917$). However, the average change in TUG times was similar between individuals with and without severe AAC. There were no significant correlations for AAC24 scores with change from baseline in handgrip strength or TUG (Table 2).

In minimally adjusted regression analyses controlling for age and treatment, and a fully adjusted model controlling for important risk factors, having severe AAC at baseline was associated with a greater decline in grip strength over 5 years (Table 3). Excluding outliers the results remained unchanged [standardised $\beta = -0.176$ (95% confidence interval: −0.337, −0.015) $p = 0.032$]. After adjusting instead for upper arm girth (as a proxy for muscle mass in the arm), the association remained significant ($\beta = -0.187$; −0.363, −0.011; $p = 0.037$). In a subgroup analysis including only women with ALM measurements and adjusting for ALM and not BMI, the association trended towards significance ($\beta = -0.226$; −0.487, 0.034; $p = 0.088$) (Table 3). For women with a relative muscle mass above the FNIH threshold ($n = 194$), AAC was associated with accelerated five-year

Table 1 Baseline demographic morphometric and functional characteristics

	All	AAC severity		p for difference
		Not severe (AAC24 ≤ 5)	Severe (AAC24 > 5)	
n (%)	1046	853 (81.6)	193 (18.4)	
Demographic data				
Age (years)	74.9 (2.6)	74.8 (2.5)	75.4 (2.7)	0.007
Smoker [n (%)]	373 (35.7)	289 (33.8)	84 (43.5)	0.032
Diabetes [n (%)]	61 (5.8)	51 (5.9)	10 (5.1)	0.669
ASVD [n (%)]	117 (11.2)	85 (9.9)	32 (16.5)	0.008
Antihypertensive use [n (%)]	449 (42.9)	357 (41.8)	92 (47.6)	0.140
Statin use (any) [n (%)]	198 (18.9)	135 (15.8)	63 (32.6)	< 0.001
Low-dose aspirin use [n (%)]	216 (20.6)	161 (18.8)	55 (28.5)	0.003
Any CV medication use [n(%)]	590 (56.4)	462 (54.1)	128 (66.3)	0.002
eGFR (mL/min/1.73 m^2)	66.9 (13.2)	66.9 (12.9)	66.6 (14.6)	0.694
Impaired renal function [n(%)]	294 (28.1)	230 (26.9)	64 (33.1)	0.084
Calcium treatment [n (%)]	510 (48.7)	422 (49.4)	88 (45.6)	0.331
Morphometric data				
Body mass index (BMI) (kg/m^2)	27.1 (4.4)	27.3 (4.6)	26.2 (3.6)	0.001
Appendicular lean mass (ALM) (kg) (n = 363)	14.9 (2.2)	14.9 (2.3)	14.8 (1.9)	0.711
Upper arm girth (cm)	32.6 (3.8)	32.8 (3.9)	32.0 (3.6)	0.013
Triceps skin fold (cm)	27.7 (8.3)	28.0 (8.5)	26.4 (7.5)	0.016
Biochemical data				
Total cholesterol (mg/dL) (n = 834)	226.1 (41.2)	225.9 (42.2)	226.7 (37.0)	0.825
Vitamin D status [n (%)]				
Deficient	34 (3.5)	27 (3.4)	7 (3.8)	0.398
Insufficient	233 (24.3)	184 (72.7)	49 (26.7)	
Normal	691 (72.1)	564 (72.7)	127 (69.4)	
Functional data				
Physical activity (kcal/kg/h)	147.1 (148.8)	147.4 (142.7)	145.9 (173.3)	0.902
Grip strength (kg)	20.7 (4.7)	20.6 (4.7)	20.7 (4.6)	0.801
Grip strength weakness [n (%)]	629 (60.1)	518 (60.7)	111 (57.5)	0.410
TUG (s)	9.7 (2.7)	9.7 (2.8)	9.4 (2.0)	0.165
Mobility impairment [n (%)]	341[32.6]	286 (33.5)	55 (28.5)	0.178

ASVD atherosclerotic vascular disease, *CV* cardiovascular, *eGFR* estimated glomerular filtration rate, *TUG* timed up and go test

declines in handgrip strength (Table 4). Additional adjustment for vitamin D status at baseline did not alter findings (Supplementary Table 1). Additional adjustment for total cholesterol attenuated the regression coefficients and trended towards significance (Supplementary Table 1).

Discussion

In this longitudinal analysis of older women who participated in a randomised controlled trial of calcium supplementation on fracture outcomes, we determined that having severe aortic calcification (measured at a single time point) was associated with 5-year declines in grip strength after accounting for multiple co-morbidities. Interestingly,

having severe AAC was not associated with 5-year declines in mobility or ALM. Overall, these findings suggest that detection of AAC in individuals, a robust indicator of cardiovascular disease and easily detectable on routine lateral spine imaging, may identify these individuals as being at a greater risk of muscle strength decline and thus may benefit from interventions to improve muscular strength. Interestingly, no association was evident regarding the association between AAC and ALM (likely due to low power) and this may suggest that any affects of AAC on muscular strength may be related more to neuromuscular factors and not muscle atrophy per se. Thus, it is unclear if vascular calcification has a direct or indirect effect, or both, on muscular strength. Aortic calcification promotes aortic stiffening which can lead to a multitude of adverse effects on peripheral blood

Table 2 Correlation of body composition and baseline functional measures with AAC as a continuous variable (AAC = 0–24)

	Spearman's rank correlation		Age-adjusted partial correlation	
	ρ	p	r	p
Baseline				
Body composition				
BMI (per SD)	−0.049	0.113	−0.067	0.030
Upper arm girth (per SD)	−0.027	0.376	−0.046	0.130
Triceps skin fold (per SD)	−0.011	0.712	−0.032	0.298
ALM at baseline (per SD) (n = 363)	−0.0293	0.577	−0.054	0.299
Functional measure				
Physical activity (per SD)	−0.006	0.835	0.005	0.865
Grip strength (per SD)	−0.007	0.818	0.015	0.609
TUG (per SD)	0.009	0.769	−0.043	0.159
Change from baseline				
Body composition				
Upper arm girth (per SD)	0.004	0.888	−0.011	0.731
Triceps skin fold (per SD)	−0.045	0.167	0.005	0.869
ALM (per SD) (n = 261)	−0.007	0.899	−0.067	0.276
Functional measure				
Grip strength (per SD) (n = 920)	−0.048	0.143	−0.055	0.094
TUG (per SD) (n = 917)	0.019	0.556	0.014	0.661

ALM appendicular lean mass, *BMI* body mass index, *SD* standard deviation, *TUG* timed up and go test

vessels and the neuromuscular system. In skeletal muscle, arteries supplying this tissue absorb most of the pulsatile energy content of propagating pressure and flow waveforms proximal to the capillaries. Sustained high central (aortic) stiffness reduces the protective stiffness gradients usually present between the heart and periphery and thus amplify the transfer of excessive, potentially harmful pulsatile energy into the periphery and tissues sensitive to high-flow and low-impedance such as the neuromuscular junction possibly impacting on efficient neuromuscular function [31]. Furthermore, occlusion of large vessels can restrict or diminish blood and thus nutrient supply to other conduit vessels hampering the proper functioning of limbs, resulting in functional decline [32]. Vascular calcification may be reflective of poor lifestyle behaviours and therefore physical decline may be inevitable but accelerated in these individuals. Other indirect factors such as circulating proteins regulating bone metabolism (for example sclerostin and Wnt signalling molecules), as well as inflammatory mediators (such as TNF-α and IL-6), may help explain the relationship demonstrated in this study as these factors are involved in both calcification and the loss of muscle mass and function [33–36]. We investigated a number of circulating factors including total cholesterol and also the vitamin D status of individuals. Adjustment for vitamin D status did not change the association; however, inclusion of cholesterol in analyses slightly attenuated the strength of the association and widened the confidence intervals, given the smaller sample size having data on cholesterol (n = 734–736/1046). Thus, these results may indicate a possibility of confounding by cholesterol, although there is no known direct physiologic connection between cholesterol and muscle strength, which would be needed to consider cholesterol a confounder. It is more likely the smaller sample size led to the slight reduction in statistical significance.

To our knowledge, no previous study has directly investigated the association of AAC and muscular strength. The present data support a previous study in older men (mean age at baseline approximately 77 years), in which greater carotid intima-media thickness (cIMT) at baseline was

Table 3 Associations between AAC severity (AAC ≥ 6 vs. < 6) and functional measure outcomes

Outcome measure	Model 1 β/Odds (95% CI)	p	Model 2 β/Odds (95% CI)	p
Baseline				
Grip strength (per SD)	0.062 (−0.091, 2.16)	0.424	0.079 (−0.080, 0.239)	0.331
Weak grip (yes/no)	0.823 (0.596, 1.134)	0.235	0.786 (0.558, 1.106)	0.167
TUG (per SD)	−0.143 (−0.298, 0.010)	0.068	−0.139 (−0.302, 0.022)	0.092
Slow TUG (yes/no)	0.746 (0.526, 1.058)	0.101	0.757 (0.519, 1.105)	0.150
ALM (per SD) (n = 363)	−0.032 (−0.285, 0.220)	0.801	0.140 (−0.083, 0.363)	0.219
Change from baseline				
Grip strength (per SD) (n = 920)	−0.178 (−0.345, −0.011)	0.037	−0.184 (−0.361, −0.008)	0.040
TUG (per SD) (n = 917)	−0.004 (−0.171, 0.163)	0.962	−0.012 (−0.187, 1.630)	0.891
ALM (per SD) (n = 261)	−0.102 (−3.670, 3.727)	0.988	0.003 (−0.271, 2.780)	0.980

Model 1 age & treatment, *2* age, treatment, BMI, smoking, ASVD, diabetes, eGFR & METS

ALM appendicular lean mass, *ASVD* atherosclerotic vascular disease, *BMI* body mass index, *CV* cardiovascular, *eGFR* estimated glomerular filtration rate, *METS* metabolic equivalents, *TUG* timed up and go test

Table 4 Fully adjusted regression models of AAC severity (AAC ≥ 6 vs. < 6) functional measure outcomes stratified by relative muscle mass

Outcome measure	Normal relative muscle mass ($n=194$) β/odds (95% CI)	p	Low relative muscle mass ($n=67$) β/odds (95% CI)	p
Baseline				
Grip strength (per SD)	0.098 (−0.069, 0.267)	0.250	−0.085 (−0.602, 0.432)	0.744
Weak grip (yes/no)	0.763 (0.534, 1.090)	0.138	1.563(0.283, 8.613)	0.608
TUG (per SD)	−0.138 (−0.290, 0.013)	0.075	−0.017 (−1.018, 0.983)	0.973
Slow TUG (yes/no)	0.764 (0.512, 1.140)	0.188	0.579 (0.162, 2.057)	0.398
Change from baseline				
Grip strength (per SD)	−0.231 (−0.418, −0.044)	0.015	0.228 (−0.312, 0.770)	0.402
TUG (per SD)	−0.014 (−0.176, 0.147)	0.861	0.098 (−1.141, 1.338)	0.875

Adjusted for Age, treatment, BMI, smoking, ASVD, diabetes, eGFR & METS

ASVD atherosclerotic vascular disease, *BMI* body mass index, *CV* cardiovascular, *eGFR* estimated glomerular filtration rate, *METS* metabolic equivalents, *TUG* timed up and go test

associated with lower handgrip strength at follow-up four years later [37]. The cIMT is a measure of atherosclerotic burden in the carotid artery and may represent both deposition of plaque on the intimal surface of the vascular wall as well as inflammatory and calcific infiltrates in the medial layer of the vascular wall [38]. No association was observed between cIMT and physical performance (determined by the short physical performance battery), or activities of daily living either at baseline or follow-up which aligns with observations from the present study. Further, despite reporting the change in cIMT from baseline to follow-up and having functional measures at baseline and follow-up, no data were reported on the association of changes in cIMT and changes in functional measures over time making it difficult to infer if worsening subclinical atherosclerosis parallels declines in physical function suggestive of a shared relationship. Another observational study of healthy middle-aged men and women (mean age approximately 47 years) has reported an inverse association between arm extensibility and the sit-and-reach test with cIMT [39]. In high-functioning middle-aged adults (mean age approximately 43 years), cIMT was higher in individuals in the lowest quartile of handgrip strength and systolic flow velocity (a measure of vessel resistance) [14]. Some literature, however, has reported no association between handgrip strength and atherosclerosis and thus the literature regarding the possible effect of vascular calcification and atherosclerosis on physical function is mixed [40]. It is interesting to note that the mean age of participants in studies reporting poor physical function as a risk factor for ASVD appears to be substantially lower than in studies reporting prevalent ASVD as a risk factor for poor physical function. That is to say that the effects of physical function on ASVD appear to become less pronounced over time (i.e. physical function contributes more to vascular risk in middle age and that in older age, prevalent vascular disease contributes to functional decline). This concept is supported by studies in autopsies of individuals 2–15 years old which have revealed that the prevalence of fibrotic/atherosclerotic lesions is as high as 20% [41]. The effects of these lesions in early life would likely accumulate such that small decrements in blood supply and sustained central stiffness impacting on neuromuscular function throughout the life course may manifest as clinically relevant functional deficits in middle age. At this point, a vicious cycle would likely ensue in which poor physical function would promote further vascular deterioration from an inability/difficulty in performing a sufficient amounts of physical exertion in a manner that would favourably evoke endothelial cell nitric oxide production, inhibit sympathetic nerve activity and ultimately allow for adequate tissue perfusion [42, 43]. Manifest vascular disease has already been demonstrated to negatively impact physical fitness and exercise capacity [44]. This may promote the progression of subclinical vascular disease to overt, clinically significant macrovascular disease due to a lack of physical activity from compromised cardio-respiratory fitness.

A previous study demonstrated that calcification in the coronary artery was associated with 9-year declines in walking speeds in older adults (mean age approximately 62 years), and in another study of older adults (mean age approximately 73 years) free of known cardiovascular disease, carotid plaque burden was associated with greater 12-month increases in TUG [15, 16]. However, in the present study we observed no association between AAC and TUG times as a continuous variable or between AAC and having mobility impairment (a TUG time > 10.2 s) at baseline or at follow-up. Also, AAC was not a significant predictor of 5-year changes in TUG. Associations of AAC may be more closely related to more discrete assessments of muscle function such as handgrip strength which involves markedly fewer muscle groups and less neuromuscular involvement.

Individuals in the present study, while not having sustained a clinical cardiovascular event, were likely to have subclinical cardiovascular disease as evidenced by the relatively high prevalence of cardio-protective medications. Exercise is commonly recommended in those with identified cardiovascular disease as part of disease management. Exercise, particularly resistance training, has proven benefits in improving muscular strength and physical function in all age groups and is safe and effective in the elderly frail [45]. Thus, those with identified AAC may benefit from muscular strength training to both improve cardiovascular risk and prevent functional decline. In a previous study, but not in this study, we identified a cross-sectional relationship between AAC severity and low relative muscle mass [12]. In this study, lean muscle mass was only available in a small subset which may have reduced power to have detected a significant association. Nevertheless, these data demonstrate that AAC may be independently related to muscle mass and function.

There are a number of important limitations to this study that must be considered when interpreting the results. Firstly, as this was an observational study, we cannot infer causality. Secondly, this study was limited by measurement of calcification at only one time point. Given an association between AAC and changes in handgrip strength was evident, it may be more informative to determine if changes in calcification correlate with changes in physical function. Given the advanced age of this cohort, it is likely that aortic calcification is already well established. Thirdly, the participants in this cohort were Caucasian women aged over 70 years and thus the results may not be generalisable to older men and non-Caucasian populations. Therefore, longitudinal analyses conducted in cohorts of men and women at an earlier age are needed to investigate if functional decline precedes the development/progression of AAC or vice versa. This is particularly important as men and women have different profiles of functional decline and cardiovascular risk during ageing. Fourth, other performance assessments, such as the 400-m walk test, may assist in clarifying the association between AAC and physical function. Fifth, DXA, while a useful tool to determine body composition, does not adequately separate out non-muscle contributors to lean mass such as fibrous tissue and water and is probably best suited to determining fat mass. Finally, we only observed modest and inconsistent associations between AAC and functional measures meaning our findings should be interpreted cautiously particularly considering some women were lost to follow-up. These women had poorer baseline functional measures meaning that we may have underestimated the true effect in this population.

In conclusion, older women with severe AAC experienced decline in handgrip strength, a measure of muscular weakness, independent of established risk factors. As such, this study supports other evidence for a potential muscle function–vascular calcification relationship. Women identified to have severe AAC may be at risk of functional decline and thus may also likely benefit from interventions aimed at promoting physical function. Future studies with longitudinal measures of AAC and comprehensive muscle phenotypes are needed to elucidate potential mechanisms. These studies should include both men and women to validate these findings.

Compliance with Ethical Standards

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Supplementary Table 1. Fully adjusted regression models of AAC severity [AAC ≥6 vs <6] and functional measure outcomes adjusting additionally for baseline vitamin D status

Outcome measure [n in analysis]	β /Odds [95% CI]	p
Baseline		
Grip strength [n=871]	0.098 [-0.067, -0.264]	0.243
Weak grip [n=871]	-0.060 [-0.142, 0.022]	0.152
TUG [n=871]	-0.116 [-0.279, 0.046]	0.161
Slow TUG [n=871]	-0.045 [-0.123, 0.033]	0.258
ALM [n=306]	0.160 [-0.062, 0.383]	0.158
Change from baseline		
Grip strength [n=763]	-0.187 [-0.372, -0.001]	0.048
TUG (per SD) [n=761]	-0.009 [-0.193, -0.174]	0.920
ALM (per SD) [n=220]	0.040 [-0.232, 0.313]	0.769

Model adjustments: Age, treatment, BMI, smoking, ASVD, diabetes, eGFR & METS

Supplementary Table 2. Fully adjusted regression models of AAC severity [AAC ≥6 vs <6] and functional measure outcomes adjusting additionally for total cholesterol

Outcome measure [n in analysis]	Total cholesterol	
	β /Odds [95% CI]	p
Baseline		
Grip strength [n=784]	0.089 [-0.086, 0.265]	0.319
Weak grip [n=784]	-0.052 [-0.140, 0.035]	0.242
TUG [n=784]	-0.088 [-0.240, 0.063]	0.252
Slow TUG [n=784]	-0.028 [-0.109, 0.051]	0.485
ALM [n=272]	0.200 [-0.032, 0.432]	0.091
Change from baseline		
Grip strength [n=736]	-0.168 [-0.358, -.020]	0.081
TUG (per SD) [n=734]	-0.047 [-0.216, 0.121]	0.579
ALM (per SD) [n=212]	0.041 [-0.246, 0.330]	0.776

Model adjustments: Age, treatment, BMI, smoking, ASVD, diabetes, eGFR & METS

Chapter 3: Associations of bone density with vascular disease and potential effects on the heart

Chapter 3.1 Introduction

There are consistent observations demonstrating associations between bone loss and the development and progression of vascular calcification [34]. Apart from co-existence, co-development and shared risk factors, there appears to be a number of common mechanisms that promote both bone loss and calcification [35]. Indeed, the bone-vascular axis has been confirmed from randomised controlled trials demonstrating that therapies with known beneficial effects in limiting bone loss can also promote the regression of vascular calcification [36].

Population data also suggest that hypertension is highly prevalent in those with low bone mass and indeed those with fractures [37, 38]. However, what is unknown is if low bone mass has some effect on heart function that may underlie the association between blood pressure and bone. In this Chapter, the effect of lower bone mass on heart function and the contribution of aortic calcification to this relationship was examined for the first time [Chapter 3.2].

Chapter 3.2: Original Research

Associations between hip bone mineral density, aortic calcification and cardiac workload in community-dwelling older Australians.

Rodríguez AJ, Scott D, Hodge H, English DR, Giles GG & Peter R. Ebeling

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SHORT COMMUNICATION

Associations between hip bone mineral density, aortic calcification and cardiac workload in community-dwelling older Australians

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Abstract

Summary In older adults, lower bone density in the proximal femur was associated with increased heart burden, and this association was linked to calcification in the aorta. These results were seen in women but not in men.

Purpose To determine whether there is an association between lower bone mineral density (BMD) and increased cardiac workload in older adults, and if this association was independent of abdominal aortic calcification (AAC).

Methods Three hundred thirty-seven participants [mean ± SD age = 70 ± 5 years and BMI = 28 ± 5 kg/m^2, 61% females] had BMD determined by dual-energy X-ray absorptiometry and AAC determined by radiography. Aortic calcification score (ACS) was determined visually in the L1-L4 vertebrae (range 0–24). Systolic blood pressure (BP) and heart rate (HR) were measured. The rate pressure product (RPP), a measure of cardiac workload, was determined by multiplying BP and HR.

Results AAC was present in 205 (61%) participants. Mean ± SD RPP was 9120 ± 1823; range was 5424–18,537. In all participants, ACS was positively associated with log-transformed RPP [LnRPP] ($\beta = 0.011$, $p < 0.001$), and severe calcification was positively associated with LnRPP ($\beta = 0.083$, $p = 0.004$ relative to no calcification). In sex-stratified analyses, these associations were significant only in females. Lower odds of any AAC were observed per 1 g/cm^2 increment in femoral neck BMD (OR = 0.08, 95% CI 0.01–0.95). A similar trend was evident in women separately (OR = 0.05, 95% CI 0–1.17) but not men. In all participants, femoral neck ($\beta = -0.20$, $p = 0.04$) and total hip BMD ($\beta = -0.17$, $p = 0.04$) were inversely associated with LnRPP after multivariate adjustment. Adjusting additionally for AAC reduced the strength of the association in femoral neck ($\beta = -0.19$, $p = 0.05$) but not total hip BMD ($\beta = -0.17$, $p = 0.04$).

Conclusion Lower BMD was marginally, but significantly with increased LnRPP, and this relationship was partially mediated by AAC suggesting that older adults, particularly females, with osteoporosis may have an increased cardiovascular risk.

Keywords Ageing · Aortic calcification · Blood pressure · Bone · Cardiovascular disease

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Introduction

Osteoporosis leads to increased skeletal fragility and susceptibility to fracture. Increasing attention has been given to the potential association and contribution of bone loss to other diseases of ageing, particularly cardiovascular diseases which

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share a number of risk factors including importantly, systemic inflammation which is key to triggering vascular injury and disrupting normal bone remodelling. Current evidence suggests that osteoporosis is associated with cardiovascular mortality and events, possibly via vascular calcification. A prospective study in post-menopausal Japanese women found that progression of aortic calcification occurred in 25% of the cohort over 9 years, and those who experienced calcification progression also experienced a 7.5% loss of metacarpal bone mass whilst those who did not experience calcification progression had only a 5.5% loss of bone mass, a clinically and statistically significant difference [1]. These data are consistent with bone loss increasing calcification and hence cardiovascular risk. As the cardiovascular risk profile differs between sexes in older age and the effects of bone loss during ageing are more pronounced in post-menopausal women, there may be some sex-specific effect of bone loss on cardiovascular disease.

Vascular calcification can have a number of functional consequences including increased cardiac workload which has the potential to lead to serious cardiovascular complications such as left ventricular hypertrophy (LVH) and myocardial infarction [2]. One simple measure of cardiac workload encompassing these outcomes is the rate pressure product (RRP). The RPP is measured as the resting heart rate (HR) multiplied by SBP. Previous studies have shown that RPP may be a better predictor of cardiovascular death than BP and is independently associated with LVH [3].

We hypothesised that, in a sample of healthy community-dwelling older adults, lower bone mineral density (BMD) would be associated with higher RPP and that this association would be linked by AAC, a surrogate measure of advanced atherosclerosis. Given differing cardiovascular risk and bone loss profiles between men and women, we further hypothesise that this relationship will be different in females and males.

Methods

Study participants

This investigation was a secondary analysis of a sub-study of generally healthy participants of the Melbourne Collaborative Cohort Study (MCCS) for which study design and inclusion/exclusion criteria have been described in detail previously [4]. Briefly, the MCCS is a prospective cohort study of 41,514 (17,045 men) community-dwelling individuals from Melbourne, Australia aged between 27 and 75 years (of which 99.3% were aged 40–69 years) at baseline (1990–94). For the present sub-study, 956 individuals who had calcium intake <500 mg/day (low) or ≥1300 mg/day (high) were approached to complete questionnaires and thoraco-lumbar radiographs in

2010 in order to examine the associations between calcium intake and cardiovascular events [5]. After exclusions, 407 participants had imaging performed, of which 353 completed whole-body DXA scans and 346 completed radiographs. A total of 337 participants completed all assessments and were included in this study (Supplementary Fig. 1). The study protocol was approved by the Cancer Council Victoria's and Melbourne Health's Human Research Ethics Committees.

Anthropometric and laboratory values

Height, weight, waist and hip circumference, waist-to-hip ratio (WHR) and body mass index (BMI) were obtained as per standard protocols. Physical activity was estimated through the Community Healthy Activities Model Programme for Seniors questionnaire which estimates total weekly energy expenditure (kJ/week).

Fasting blood samples were collected. High-density lipoprotein (HDL), serum homocysteine (Hcys) and calcium (Ca) were measured by technicians at Melbourne Pathology centres. Serum vitamin D levels were analysed at the RMIT Drug Discovery Technology laboratories (Melbourne, Australia) using liquid chromatography-tandem mass spectroscopy.

Bone density and body composition analysis

Bone densitometry and body composition analysis were performed using a Hologic densitometer (QDR 4500 W, Hologic Inc., Bedford, Massachusetts). Standard operating procedures were followed for participant positioning and acquisition. Scans were analysed using Hologic APEX 3.1 software according to standard procedures. Femoral neck BMD was measured as this site is relevant to fracture prediction, further total hip BMD was measured as cortical thickness in the trochanter is relevant to fracture.

Cardiovascular assessment

Resting systolic (SBP) and diastolic (DBP) blood pressure and heart rate (HR) were measured in a seated position using an automatic blood pressure monitor (Omron Healthcare HEM907, Sydney Australia) (mmHg). Two readings of blood pressure were taken, separated by a 5 min interval. Hypertension was defined as a systolic blood pressure >140 mmHg according to the 2011 consensus of the American College of Cardiology and American Heart Association.

We calculated the rate pressure product (RPP), as HR × SBP (units: bpm × mmHg). The RPP gives an indication of cardiac workload, where a higher RPP corresponds to a higher cardiac workload. Threshold values are defined as "high" = >30,000; "high-intermediate" = 25,000–29,999; "intermediate" = 20,000–24,999; "low-intermediate" = 15,000–19,000 and "low" = <14,999 [6]. Even low RPP has

sensitivity in detecting "silent" ischemia and may indicate clinically relevant disease.

AAC deposits were assessed on thoraco-lumbar radiographs and independently scored using a visual technique by two trained operators according to the extent and severity of calcified deposits in the anterior and posterior walls of the abdominal aorta at the level L1-L4 vertebrae in a method described previously [7]. The final aortic calcification score (ACS) was a composite of all four vertebral segments ranging from a minimum "0" to a maximum of "24". Participants were stratified into AAC severity groups, defined as "no calcification" (ACS = 0), "moderate" (ACS between 1 and 5) and "severe" (ACS score ≥6).

Statistical analysis

Variables were reported as mean ± standard deviation (SD), median and interquartile range (IQR) as appropriate to distribution. Categorical variables were reported as frequency and percentage. The natural logarithm was taken for the values relating to caloric expenditure (LnCaloric) and RPP (LnRPP). Comparison of variables between sexes were performed using Student's t-test, Mann-Whitney U-test or χ^2 test as appropriate. Age-adjusted partial correlations between ACS and RPP were made using Pearson's correlation. For the association between calcification and LnRPP, a linear regression model was fitted adjusting for age, sex, percentage body fat, caloric expenditure, serum calcium, homocysteine, HDL cholesterol, vitamin D and smoking status (non-smoker or ever-smoked). Blood pressure or hypertension were not included as co-variates due to collinearity with the LnRPP variable. For the association of calcification severity with LnRPP relative to no calcification, an ordinal regression model was fitted with adjustment as above. For the associations between femoral neck or total hip BMD and AAC, logistic regression models were fitted with adjustment (including hypertension). The associations between femoral neck or total hip BMD and the severity of calcification were determined by ordinal regression with adjustment (including hypertension). The odds derived from the ordinal regression results represent the likelihood of being in a higher category of ACS for increases in the exposure (i.e., BMD). For the associations between femoral neck or total hip BMD and LnRPP linear regression were fitted with adjustment as above (excluding hypertension). To determine what effect calcification had on this association, models were fitted with and without AAC as a co-variate. Mediation analysis was used to examine the mediated effect of calcification on RPP [8]. A series of linear regression models were fitted. The first model tests the hypothesis that BMD affects LnRPP, as the "direct" effect. The second model tests the hypothesis that BMD affects AAC which in turn affects LnRPP. From these models, we could test the "indirect" or mediated effect of AAC on LnRPP. The

beta-coefficients in the second model were multiplied together, and the percentage of mediated effect relative to the direct effect was calculated. Given our hypothesised differences between men and women, we conducted sex-specific analyses irrespective of whether significant sex-by-variable interactions were found as the power for detecting interactions was limited. Regression diagnostic plots are also provided (Supplementary Fig. 2). Values were considered significant if $p < 0.05$, and all data was analysed using SPSS Statistics v22 (IBM, Armonk, NY, USA) by AJR and checked for accuracy by DS.

Results

Of the 337 participants included, 205 were women (approximately 61%). The mean age was 70.5 years, and mean BMI was in the overweight range (mean 28.1 kg/m^2 ± 5.1), with no significant difference in age and BMI between sexes (Table 1). Women had significantly higher serum concentrations of HDL, calcium, vitamin D and a significantly greater proportion of individuals with hypertension and any AAC (65% compared with 58% in men). Men had significantly greater waist circumference, waist-to-hip ratio, SBP, DBP, total hip and femoral neck BMD. There were no other sex-specific differences (Table 1).

Age-adjusted partial correlation ACS and RPP was ($r = 0.229$, $p < 0.001$) probably due to the blood pressure nature of RPP. As such, the partial correlation between SBP and ACS was ($r = 0.263$, $p < 0.001$) which is in line with orthodox understanding. Higher ACS was independently associated with higher LnRPP in the whole sample ($\beta = 0.011$; 95% CI 0.006, 0.016; $p < 0.001$). A sex-by-ACS interaction term was not significant ($p = 0.099$). However, in sex-specific analyses, the ACS was positively and independently associated with LnRPP in females ($\beta = 0.015$; 95% CI: 0.008, 0.021; $p < 0.001$) but not in males ($\beta = 0.003$; −0.007, 0.013, $p = 0.527$). Furthermore, participants with severe, but not moderate, calcification had significantly higher LnRPP relative to those with no calcification ($\beta = 0.083$; 95% CI 0.026, 0.140; $p = 0.004$), although sex-stratified analyses demonstrated this association was present only in females (0.155; 0.042, 0.188; 0.002).

There was a statistically significant reduction in the odds of having any AAC in participants with higher femoral neck BMD (OR = 0.08 95% CI 0.01, 0.95) overall. A sex-by-BMD interaction term was not significant ($p = 0.432$), and sex-stratified analyses revealed no association between femoral neck BMD and any AAC, although the association for women approached significance (Table 2). There was no association between total hip BMD and any AAC in the whole cohort, or for men or women separately. In ordinal models, a unit increase in femoral neck BMD was associated with a

Table 1 Descriptive characteristics of participants

	Total	Men	Women	p
N (%)	337	132 (39.1)	205 (60.9)	n/a
Age (years)	70.5 ± 5.4	70.3 ± 5.4	70.7 ± 5.5	0.569
BMI (kg/m^2)	28.1 ± 5.1	28.1 ± 4.2	28.1 ± 5.6	0.926
Body fat (%)	33.7 ± 9.9	29.1 ± 6.1	44.9 ± 6.4	<0.01
WC (cm)	83.1 ± 12.7	91.4 ± 11	77.7 ± 10.8	<0.01
WHR	0.82 ± 0.09	0.91 ± 0.06	0.77 ± 0.06	<0.01
SBP (mmHg)	133.8 ± 16.2	135.8 ± 15.3	132.5 ± 16.7	<0.01
DBP (mmHg)	76.2 ± 10.8	81.22 ± 9.1	73.0 ± 10.5	<0.01
HTN [n(%)]	106 (31.6)	47 (35.9)	59 (28.9)	<0.01
Smoking [n(%)]	145 (43)	67 (50.8)	78 (38)	0.361
HDL (mmol/L)	1.6 ± 0.4	1.4 ± 0.3	1.7 ± 0.4	<0.001
HR (bpm)	68.0 ± 10.0	67.1 ± 12.1	68.5 ± 8.4	0.208
RPP (SBP×HR)	9120 ± 1823	9128 ± 1975	9116 ± 1723	0.852
RPP range (min-max)	5424–18,537	5444–18,637	5533–14,960	n/a
LnRPP	9.1 ± 0.2	9.1 ± 0.2	9.1 ± 0.2	0.915
Total hip BMD (g/cm^2)	0.925 ± 0.143	0.998 ± 0.124	0.878 ± 0.135	<0.001
Femoral neck BMD (g/cm^2)	0.743 ± 0.115	0.787 ± 0.108	0.714 ± 0.111	<0.001
Hcys (mmol/L)	10.2 ± 2.6	10.5 ± 2.3	10.0 ± 2.7	0.072
Ca (μmol/L)	2.4 ± 0.1	2.3 ± 0.1	2.4 ± 0.1	0.002
High Ca intake [n(%)]	170 (50.4)	70 (53.0)	100 (48.8)	0.503
Vitamin D (ng/mL)	75.0 ± 26.3	67.2 ± 25.1	79.9 ± 25.9	<0.01
ACS	3.57 ± 4.22	3.68 ± 3.92	3.50 ± 4.41	0.704
AAC [n(%)]	205 (60.8)	86 (65.2)	119 (58)	0.021
Caloric expenditure (kJ/week)	33.3 ± 3.30	33.4 ± 3.30	33.1 ± 3.05	0.147

BMI body mass index, *WC* waist circumference, *WHR* waist-to-hip ratio, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *HTN* hypertension, *TC* total cholesterol, *LDL* low density lipoprotein, *HDL* high density lipoprotein, *HR* heart rate, *RPP* rate pressure product, *LnRPP* natural logarithm of RPP, *BMD* bone mineral density, *Hcys* homocysteine, *Ca* calcium, *ACS* aortic calcification score, *AAC* any abdominal aortic calcification

reduced likelihood of having more severe calcification relative to having no calcification, and this tended toward significance (Table 2). In a sex-stratified analysis, there was no association between femoral neck BMD and calcification severity in males but the association tended toward significance in females (Table 2). There was no association between total hip BMD and calcification severity in the whole cohort, or for men or women.

Higher femoral neck and total hip BMD were independently associated with lower RPP in the whole sample (Table 2). Sex-by-BMD interaction terms were not significant ($p = 0.584$ and 0.735, respectively). With further adjustment for presence of any AAC, the coefficients were reduced, and the association between femoral neck BMD and RPP was no longer significant. There was no association for femoral neck or total hip BMD with RPP in sex-stratified analyses. In mediation analysis for the whole cohort, the percent mediated effect of aortic calcification was approximately 13.5% for femoral neck BMD and approximately 15.2% for total hip BMD. In males, these values were 7.6 and 8.4% for femoral neck and total hip BMD, respectively; and

in females these values were 21.0 and 23.1% for femoral neck and total hip BMD, respectively (Table 2).

Discussion

We determined that higher femoral neck BMD was associated with a statistically significant lower odds of having any AAC, and there was a trend for significance for severe AAC relative to no calcification, and that BMD at the femoral neck and total hip was inversely associated with RPP in women only. Furthermore, adjustment for AAC appeared to attenuate the association of BMD with RPP. These findings are clinically meaningful as increased cardiac workload has the potential to cause the rupture of vulnerable plaques leading to serious outcomes such as ST segment depression embolism and stroke.

To our knowledge, this analysis is the first investigation to demonstrate that lower BMD in the proximal femur was associated with higher cardiac workload, and this association

Table 2 Regression models and mediation analysis for the association of BMD with the outcomes LnRPP (top panel) and AAC (middle panel)

LnRPP model		Femoral neck BMD			Total hip BMD		
		β	95% CI	p	β	95% CI	p
1	All	−0.20	−0.39, −0.01	0.040	−0.17	−0.33, −0.01	0.040
	Male	−0.25	−0.57, 0.06	0.117	−0.18	−0.46, 0.09	0.195
	Female	−0.17	−0.42, 0.07	0.178	−0.15	−0.36, 0.04	0.133
2	All	−0.19	−0.39, 0.00	0.051	−0.16	−0.33, −0.01	0.049
	Male	−0.25	−0.58, 0.07	0.120	−0.18	−0.46, 0.10	0.199
	Female	−0.15	−0.40, 0.11	0.250	−0.14	−0.35, 0.07	0.180
AAC model		OR	95% CI	p	OR	95% CI	p
Any	All	0.08	0.01, 0.95	0.046	0.22	0.03, 1.59	0.133
	Male	0.23	0.00, 12.21	0.472	0.36	0.01, 11.48	0.566
	Female	*0.05*	*0.00, 1.17*	*0.063*	0.16	0.01, 2.01	0.155
Severe	All	*0.13*	*0.02, 1.11*	*0.062*	0.27	0.05, 1.55	0.141
	Male	0.98	0.03, 33.24	0.991	0.98	0.05, 19.81	0.991
	Female	0.06	0.00, 1.01	0.051	0.16	0.02, 1.54	0.113
		Effect	Path	β	Effect	Path	β
Mediation analysis	All	Total	BMD vs. RPP	−0.19	Total	BMD vs. RPP	−0.16
		Indirect	(BMD vs. ACS)x (ACS vs. RPP)	−0.027	Indirect	(BMD vs. ACS)x (ACS vs. RPP)	−0.026
		Percent mediated effect		13.5%	Percent mediated effect		15.2%
	Male	Total	BMD vs. RPP	−0.25	Total	BMD vs. RPP	−0.18
		Indirect	(BMD vs. ACS)x (ACS vs. RPP)	0.01914	Indirect	(BMD vs. ACS)x (ACS vs. RPP)	0.01523
		Percent mediated effect		7.6%	Percent mediated effect		8.4%
	Female	Total	BMD vs. RPP	−0.15	Total	BMD vs. RPP	−0.14
		Indirect	(MD vs. ACS) x (ACS vs. RPP)	−0.0315	Indirect	(BMD vs. ACS)x (ACS vs. RPP)	−0.0323
		Percent mediated effect		21.0%	Percent mediated effect		23.1%

Model 1 adjusted for Age, sex, percentage body fat, caloric expenditure, smoking, Hcys, calcium, vitamin D, HDL

Model 2 adjusted for Model 1 + presence of AAC

was attenuated when AAC (a surrogate marker of advanced atherosclerosis) was included in the model, suggesting that AAC is a mediator of the association. Indeed, in mediation analysis, one tenth of the variance in RPP was attributable to calcification, and in females this increased to over one fifth, highlighting a potential sex-specific difference. Osteoporosis and cardiovascular disease share common risk factors and are associated with ageing. Having a hip T-score ≤2.5 was associated with a hazard ratio of 2.1 (1.2–3.6) for cardiovascular events [9], and hip osteoporosis was associated with advanced atherosclerosis in elderly women [10]. In cross-sectional studies, lower spine (−1.63) and femoral neck (−1.34) T-scores were observed in those with atherosclerotic plaques in the common carotid artery versus those that did not have plaques (−1.38 and −0.75, respectively) [11]. In Korean men and women, ultrasound determined heel BMD explained approximately 25% of the variance in left ventricular mass [12].These studies suggest cardiovascular risk is associated with bone loss in older people. Further, the increased cardiovascular risk seemed to be driven by factors associated with increased cardiac workload as left ventricular hypertrophy, and sub-clinical atherosclerosis are associated with cardiac insufficiency [2]. Our data supports this notion as we demonstrated an independent association between lower femoral neck and total hip BMD with RPP. Furthermore, the coefficient of the association between BMD (femoral neck or total hip) and RPP was attenuated, and the model lost significance after adjustment for calcification.

There is an inverse relationship between bone density and vascular calcification. One mechanism explaining this relationship is the conversion of vascular smooth muscle cells into bone-like cells which involves matrix-metalloproteinase modulation by homocysteine and transactivation of the RunX promoter [13, 14]. The Framingham Heart Study demonstrated that with progressive metacarpal bone loss, there was a concomitant increase in aortic calcification in men and women

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[15]. In addition, changes in bone density predicted progression in vascular calcification [1]. Data from the present study supports this conclusion as we established that relative to an individual with a lower femoral neck BMD, having a 1 g/cm^2 higher BMD was associated with a 92% reduced odds of having any AAC. Importantly anti-resorptives can limit the progression of aortic stenosis (characterised by aortic valve calcification) [16], suggesting that slowing bone loss concomitantly slows calcification and by extension, interventions which target bone mass may be associated with improvements in cardiovascular risk. Calcification in major arterial beds was associated with increased risk of all-cause and cardiovascular mortality in a 6-year-follow-up study of healthy Dutch men and women [17]. One of the mechanisms by which advanced atherosclerosis increases cardiovascular risk may be through increasing cardiac burden as calcified vessels loose distensibility. In a rodent model of aortic calcification, overexpression of parathyroid hormone leads to the reversal of aortic calcification, reduction in collagen content and wall thickness resulting in improved aortic distensibility, suggestive of calcification disturbing the elastic properties of the aorta [18]. Data from the present study support this understanding as ACS was linearly associated with RPP. We further established a role for aortic calcification in potentially mediating the association between bone density and RPP.

These associations were more consistent in women than in men, suggesting that the effects of bone loss on cardiovascular risk may be driven by sex-steroids and their deficiency. Postmenopausal women are at a substantially increased risk of osteoporosis and exhibit more rapid and pronounced bone loss during ageing. Additionally, women had a fivefold increased risk of having a high aortic valve calcification load compared to men suggesting a clear sex difference [19] most likely due to oestrogen loss. Estradiol therapy in a randomised trial was successful in reducing subclinical atherosclerosis, suggesting a direct role for oestrogen-containing hormones in cardiovascular disease in older females [20]. Overall, our data supports these observations as the association of BMD with RPP was largely present in women and not in men.

This study was limited by a cross-sectional design which cannot imply causation and small sample size which limits generalisability. Generalisability is further limited by participants having been recruited on the basis of having low (<500 mg/day) or high (≥1300 mg/day) calcium intake representing the extremes of calcium intake which may introduce some selection bias to the sample. Participants had low RPP scores, and thus these results may not apply to different populations. No study to date has examined the effect of calcium intake on RPP. Future studies are required to validate these findings in individuals with normal calcium intake. The present analysis was based on a small sample, and gender stratified analyses further decreased the sample size reducing power to detect significant associations. In conclusion,

community-dwelling older adults with low BMD have higher cardiac workload which may be partly explained by higher vascular calcification. Further research is required to understand the precise mechanistic pathways involved in bone loss that contribute to increased vascular calcification and the functional consequences of this, including increasing cardiac workload.

Compliance with ethical standards

Conflict of interest   None.

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Total participants in MCCS
41, 514
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↓
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Participants with high/low calcium intake
invited to complete questionnaires and
radiography/densitometry
956
```
→
```
Responded to invitation
759
```
→
```
Satisfied inclusion criteria
652
```
↑
```
Participants excluded due to use of
medications known to effect bone and mineral
metabolism
107
```
→
```
Had imaging performed
407
[352 DXA, 346 Radiographs]
```
↑
```
Participants excluded due to ineligibility or
refused further participation
245
```
→
```
Participants included
337
```
↑
```
Participants excluded due to missing data
15
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136

Supplementary Figure 2. Regression diagnostic plots for regression of femoral neck bone density and RPP in females (above) and in males (below).

Chapter 4: Associations of vitamin D supplementation and calcium intake on cardiovascular disease markers and outcome

Chapter 4.1 Introduction

Current recommendations for maintaining adequate bone and muscle health throughout older age include obtaining 1000-1300mg/day of calcium (preferentially from dietary sources as these sources contain protein and other minerals required in a balanced and healthy diet) and obtaining 800-1000IU/day of vitamin D to achieve a blood vitamin D level of at least 50nmol/L at the end of winter or early spring. In Australia, during summer time, only 5-10 minutes of sun exposure is required for those with fair skin and 15-60 minutes for those with darker skin. In winter, the times required increase to 7-30 minutes and 20-180 minutes for fair and darker skin, respectively [40]. In Australia, vitamin D supplements are available over the counter at a maximum concentration of 7,000IU, or 50,000IU by prescription for those that are unable to get enough sun exposure.

There is robust evidence as to the musculoskeletal benefit of these micronutrients, particularly in individuals with deficiency [41]. Despite this, there is uncertainty as to the cardiovascular benefit and safety of calcium intake and vitamin D supplementation. This is despite the fact that low calcium intakes and vitamin D deficiency are associated with increased cardiovascular disease and that

musculoskeletal diseases commonly co-exist with cardiovascular diseases in older age [42].

This Chapter presents three studies addressing important questions in this field. As we now understand, much of cardiovascular disease risk is related to 'non-traditional' risk factors, that is, factors other than blood pressure, serum lipids and adiposity. One of these risk factors is arterial stiffness which has been demonstrated to improve risk stratification [43]. Older adults with low vitamin D have increased arterial stiffness [44] but the effects of supplementation on improving this outcome have yet to be clarified and this is addressed in Chapter 4.2. Further, the pathophysiological basis for arterial disease is partly attributable to increases in inflammation [45]. Vitamin D has known immunomodulatory effects yet its effects in addressing the underlying causes of arterial disease (inflammation) in the setting of established cardiovascular disease is yet to be fully established and this was explored in Chapter 4.3. Finally, there has been interest in understanding the cardiovascular safety of calcium supplementation as some reports have suggested that certain cardiovascular events increase [46]. For the practicing clinician this means recommending obtaining calcium from dietary sources and thus attention has turned to understanding if any supposed deleterious effects of calcium supplements is also seen at the upper extremes of intake values [Chapter 4.4].

Chapter 4.2: Systematic Review

Effect of vitamin D supplementation on measures of arterial stiffness: a systematic review and meta-analysis of randomized controlled trials.

Rodríguez AJ, Scott D, Srikanth V & Ebeling PR

Effect of vitamin D supplementation on measures of arterial stiffness: a systematic review and meta-analysis of randomized controlled trials

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Summary

Background Low vitamin D has been associated with poor arterial compliance in observational studies. Arterial stiffness has prognostic value for cardiovascular disease risk. The aim of this systematic review was to clarify the literature surrounding the use of vitamin D to ameliorate arterial stiffness.

Methods We conducted a systematic review of the MEDLINE, Scopus and EMBASE databases for randomized controlled clinical trials investigating the effect of vitamin D supplementation on pulse wave velocity (PWV) and/or augmentation index (AI) as indicators of arterial stiffness. We meta-analysed data and calculated standardized mean difference (SMD) and 95% confidence intervals (CI) using inverse-variance models on RevMan v5.3 software. Study quality was assessed using a modified Jadad scale.

Results A total of 607 unique records were identified, of which 18 satisfied our inclusion and exclusion criteria. Study quality was high, ranging from 9 to 12 (of 13). Study design in terms of vitamin D dosing protocol (range: 1000–5700 IU/day), follow-up times (range: 1–12 months), sample size (range: $n = 29$–183) and recruitment strategies varied markedly. Thirteen studies had data for meta-analysis. Vitamin D was associated with nonsignificant reductions in PWV [SMD = -0.10; 95% CI: -0.24, 0.04; $P = 0.17$; $n = 806$ from ten studies] and AI [-0.15; -0.32, 0.02; 0.08; $n = 551$ from eight studies].

Discussion There is inconsistent evidence to suggest that vitamin D supplementation improves indicators of arterial stiffness. This may be attributable to the heterogeneity in study design. Therefore, large and well-designed randomized studies are required to determine the casual relationships between vitamin D and arterial stiffness and cardiovascular risk.

Introduction

Vitamin D is a fat soluble steroid hormone with pleiotropic effects. Principally, vitamin D regulates calcium homeostasis and mineral metabolism by influencing intestinal absorption, bone resorption and renal retention. Vitamin D also has a number of nonskeletal effects that may favourably influence the cardiovascular system such as downregulation of the renin–angiotensin system,[1] enhancing insulin sensitivity[2] and modulating inflammation.[3] Previous clinical studies have indicated that low vitamin D (defined as serum calcifediol/25-hydroxyvitamin D (25OHD) concentration below 50 nmol/l [=20 ng/ml] as according to The Endocrine Society guidelines[4]) can impair vascular function which may compromise vascular compliance (the elastic property of blood vessels) manifesting as increased arterial stiffness.[5]

Increased arterial stiffness is a marker for atherosclerotic diseases and is associated with a number of other important clinical outcomes including increased calcification,[6] decreased bone mineral density,[7] decreased muscle strength and increased falls risk[8] and reduced quality of life.[9] Pulse wave velocity (PWV) is a simple, robust and validated measure of arterial stiffness.[10] In brief, the arterial pressure wave form is a composite of the forward pressure wave created by ventricular contraction and a reflected wave from a distal site.[10] The gold standard measurement of arterial stiffness is the carotid–femoral PWV. This is estimated using the foot-to-foot velocity method whereby transcutaneously, the right common carotid artery and the right femoral artery and the time delay (or transit time, Δt) are measured (in seconds, s) between the feet of the two waveforms (Fig. 1). A

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645
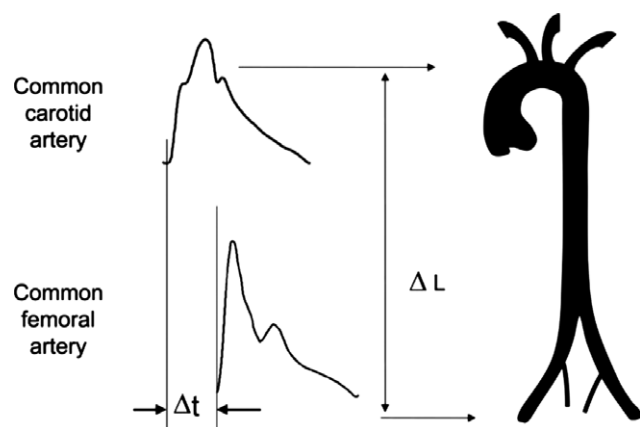
Fig. 1 Carotid–femoral PWV measurement using the foot-to-foot method.[11]

variety of different waveforms can be used including Doppler, pressure and distension. The distance (L) covered by the waves between these two sites is measured (in metres, m), and PWV is then calculated as ¼ L/Δt with the unit m/s.[11] The less compliant the arteries, the faster the reflected wave returns augmenting systolic pressure interpreted as an increased PWV. The extent of this augmentation in systolic pressure is called the augmentation index (AI).

Observational studies have suggested that vitamin D deficiency or insufficiency is associated with a poorer vascular profile including increased stiffness.[12] No causal relationship has been established. Therefore, naturally, this has stimulated research into improving vitamin D status to determine the impact of vitamin D supplementation on these end-points (PWV and AI). This review will critically analyse randomized controlled studies that utilized vitamin D supplementation and assessed the effect of this intervention on the outcome of PWV and/or AI. We aimed to clarify what effect vitamin D supplementation has on these end-points by way of systematic review and where appropriate data were available, meta-analysis.

Methods

Study focus and eligibility criteria

We conducted a systematic review and meta-analysis according to the guidelines outlined in the PRISMA statement (Preferred Reporting Items for Systematic Reviews and Meta-Analyses).[13] We sought original randomized controlled clinical trials that assessed the effects of vitamin D supplementation versus control on the outcome of PWV and/or AI in samples of patients with any pathology. Studies were eligible if the trial was randomized in design, compared vitamin D supplementation to a placebo or control drug and reported descriptive statistics for PWV and/or AI before and following the intervention. Specific exclusion criteria were as follows: studies that were not randomized in design, studies that did not use vitamin D as the treatment arm and studies that did not report outcome of PWV or AI following

vitamin D treatment (i.e. reported some other measure of arterial stiffness) and animal- or cell-based studies.

Literature search

Relevant studies were retrieved from the MEDLINE (archives from 1966 to 2014), Scopus (1996–2014) and EMBASE (1947–2014) databases by applying a search strategy which broadly followed this protocol: 'vitamin d' [Title/Abstract] AND ['pulse wave velocity' (Title/Abstract) OR 'augmentation index' (Title/Abstract)] with no language restriction on the 16 of May 2015. A detailed search protocol is provided in supplementary materials. Titles and abstracts of identified records were screened. Additionally, we manually scanned the reference lists of eligible texts and the related articles lists that were generated, following a database search for other potential studies of interest. We termed these texts the 'grey literature'. Following title and abstract screening, the full-text manuscripts were evaluated to determine eligibility. For conference abstracts and other eligible studies otherwise unavailable online as a full-text manuscript, attempts were made to obtain the full-text manuscripts direct from authors and further, and data were also sought direct from authors in order to ensure study eligibility and to complete the data set for comparison and review.

Data capture and presentation

Data were extracted by a single reviewer (AJR) with the aid of an extraction template. Specifically, we sought information relating to study design, sample demographics, vitamin D regimen, outcome definition and assessment, statistical analyses employed and limitations highlighted by the authors. These data were then tabulated into a format that allowed comparison between trials of the pertinent aspects of the study, namely study design, patient demographics, and effect of vitamin D on study endpoints.

Quality assessment

In addition to data extraction, we performed a quality assessment of included studies. As no standardized quality assessment tool exists for randomized trials of vitamin D on the outcome of PWV or AI, we modified the previously validated Jadad scale to suit our aims.[14] Using a semiquantitative method, each included study was judged in the following areas: study design: sample size and representativeness, outcome definition and assessment, comparability of results, and statistical methods. The maximum possible score that could be achieved was 13 and a score of less than 9 was considered low quality and excluded from analysis.[14] A sample data extraction form and quality assessment tool is provided in Supplementary Materials.

Statistical analysis

Meta-analysis eligibility. Studies were eligible for meta-analysis if they first satisfied the inclusion and exclusion criteria for
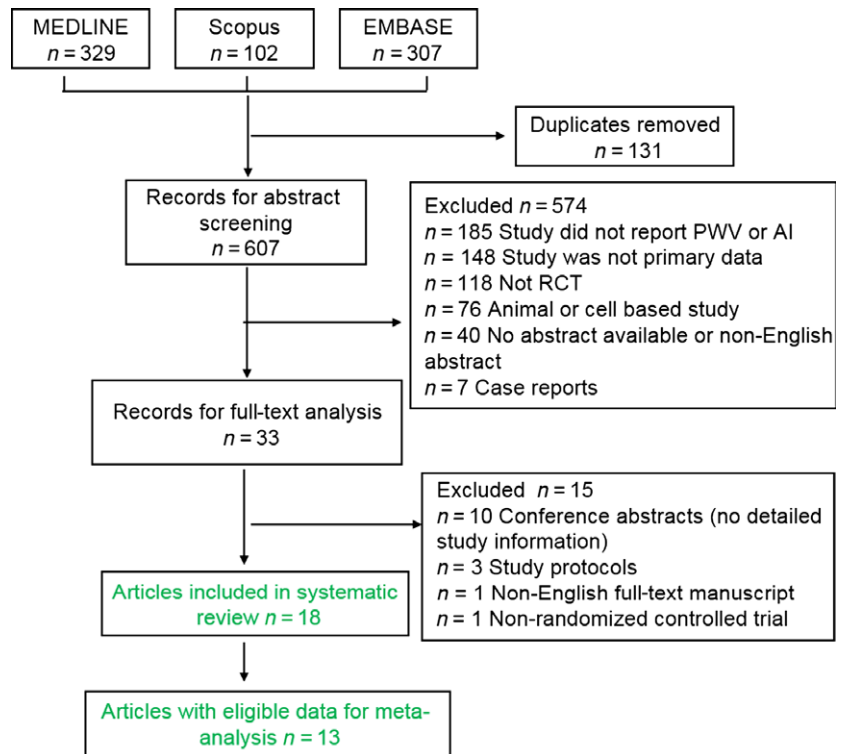
Fig. 2 Study selection flow diagram.

systematic review and reported mean baseline and follow-up data for PWV and/or AI in groups of participants receiving vitamin D (experimental) and placebo (control). Studies were excluded from meta-analysis in instances where the control group did not receive a placebo but instead a lower dose vitamin D.[15,16] For studies that did not specifically report data that we were looking to synthesize into the meta-analysis model [e.g. studies that reported change from baseline to follow-up rather than means at baseline and follow-up, $n = 2$[17,18]], contact was made with authors requesting these specific data in an attempt to include as much literature as possible but replies were not all forthcoming.

Meta-analysis. Data were first tabulated into a format that allowed comparison between mean baseline and follow-up data for PWV and AI comparing groups of patients who received vitamin D relative to patients who received placebo. These data were then synthesized using an inverse-variance method to determine the standardized mean difference (SMD), and 95% confidence interval (95% CI) was calculated. Heterogeneity was determined by the inconsistency percentage (I^2) statistic where a random effects model was applied in analyses where I^2 was greater than 50%.[19] Sensitivity analyses restricting the operation to studies employing an equivalent daily dose vitamin D of ≥3000 IU (arbitrarily defined), studies with a follow-up of ≤3 months, studies with a follow-up of ≥3 months (arbitrarily defined), studies involving chronic kidney disease patient, studies involving patients recruited due to hypertension, studies involving patients with insulin resistance (type 2 diabetes [T2D] and polycystic ovarian syndrome [PCOS]), studies with a mean sample age <55 and studies with a mean sample age ≥55. All

statistical operations were performed using RevMan v5.3 software (The Nordic Cochrane Centre, The Cochrane Collaboration 2012).

Results

Literature search

The initial database search yielded 783 eligible records, of which 131 were duplicates (that is, appearing in more than one database) leaving 607 unique records for abstract screening. Following screening, a further 574 records were excluded primarily because the study did not assess an outcome of interest, namely PWV or AI (number of records excluded on this basis, $n = 185$). This left 33 records for full-text review and of these 15 records were excluded mainly because the record was a conference abstract and not a full manuscript ($n = 10$ records) leaving an overall 18 full-text manuscripts included as part of the systematic review and of these, 13 records were included in the meta-analysis as they had eligible data to do so[20–32] (Fig. 2). One author of a conference proceeding provided baseline data but no follow-up data meaning these results could not contribute to our analyses [personal communication, can be provided on request]. Other authors who were contacted directly for their abstract proceedings did not respond to our communications.

Included Studies

All 18 studies were randomized controlled trials, of which 17 were double-blinded and one other was open label.[15] All studies were placebo-controlled except for two studies,[33,16] which

compared the effects of a high versus low-dose vitamin D supplementation as the trial arms; therefore, 15 studies were eligible for meta-analysis. Study population size varied from a minimum $n = 29$ participants[22] to maximum $n = 183$ participants.[28] Recruitment based on deficient or insufficient vitamin D status (10–60 ng/ml) was part of trial inclusion criteria in thirteen studies.[16–18,22,24–26,28–33] Study samples were heterogeneous; four studies were conducted in patients with chronic kidney disease (CKD),[22,24,25,27] four studies were conducted in patients with hypertension,[21,26,28,31] three studies were conducted in patients with T2D,[18,20,32] two studies were conducted in postmenopausal women,[17,33] and other studies were conducted in populations of black youths,[15] older community-dwelling individuals,[16] patients with PCOS,[23] patients with chronic fatigue syndrome (CFS) [30] and peripheral artery disease (PAD).[29] As expected, the inclusion and exclusion criteria for these studies varied considerably and this is summarized in Table 1. All studies reported using vitamin D3 (cholecalciferol) as the vitamin D supplement. Dosing regimens varied considerably. As aforementioned, we defined an equivalent daily dose which ranged from a minimum 1000 IU/day[20] to a maximum 5700 IU/day.[25] One study compared two different single high-dose vitamin D supplementation regimens.[16] Follow-up times varied between a minimum of 2 months[16,25] and maximum of 12 months.[20,31] Six studies had changes in PWV as a study end-point,[15,22,24,28,30,32] four studies had AI as a study end-point,[20,26,29,33] and eight studies had both PWV and AI as study outcomes.[16–18,21,23,25,27,30] Of the studies that had PWV as an outcome, the majority of them assessed either carotid–femoral PWV[16,17,23,27,28,30] or carotid–radial PWV.[15,20,26,29,31] These data are summarized in Table S1.

Literature quality

Using a modified Jadad scale of randomized controlled trial study quality, scores ranged between 9[24,25,33] and 12[23] of a possible maximum of 13 (Table S2), and thus, all were included in further analyses. All studies reported randomization, inclusion and exclusion criteria, outcome measures, intervention description, control groups and statistical methods. No study reported sample size justification by way of a power calculation or other method. Adverse events and other safety data were reported in only one study.[23]

Patient demographics

Mean age (in years) ranged from $16·5 ± 1·4$[15] to $79·3 ± 7·0$[16] in patients receiving vitamin D (experimental) and from $16·3 ± 1·1$[15] to $80·5 ± 6·6$[16] in patients receiving control treatments (control). Three studies had samples entirely female.[17,23,33] In other samples, the proportion of males ranged from 19%[25] to 68%[27] in experimental patients and ranged from 20%[25,30] to 73·7%[22] in controls. Mean body mass index (BMI) in kg/m^2 ranged from $24·0 ± 4·5$[27] to $32·4 ± 6·4$[33] in experimental patients and ranged from $23·8 ± 4·4$[27] to $33·3 ± 7·3$[33] in control patients. These data and other important patient characteristics are summarized in Table 2.

Baseline and follow-up vitamin D

Thirteen studies recruited participants specifically with vitamin D inadequacy (deficiency or insufficiency) defined according to their study protocol (Table S3).[16–18,22,24–26,28–33] Twelve studies measured serum 25-OH vitamin D.[16–18,20,23,24,26,28–31,33] Four studies measured plasma 25-OH vitamin D,[15,21,25,27] and two studies did not specify this information.[22,32] Eleven studies provided follow-up circulating vitamin D concentrations in case and control groups to enable comparison.[16,18,20,23,25–28,30,32,33] For studies that reported group differences in 25-OH vitamin D at baseline (pretreatment), there were no significant differences in mean concentrations between experimental and controls groups. In studies that reported follow-up vitamin D concentrations only one study did not record differences between experimental and control groups.[20] Eight studies reported significantly higher 25-OH vitamin D in experimental groups compared to controls.[16,18,23,25,27,28,30,32] Further, only two studies reported not achieving mean vitamin D adequacy (>20 ng/ml) in the experimental groups following intervention.[16,20] All these data are reported in Table S3.

Pulse wave velocity

In all studies at baseline, there were no significant differences in PWV between experimental and control groups (Table 3). Specifically, in experimental patients, PWV velocity (m/s) ranged from $5·41 ± 0·73$ (measured as carotid–femoral)[15] to $18·97 ± 3·38$ (carotid–brachial),[32] and in control patients, it ranged from $5·38 ± 0·53$ (measured as carotid–femoral)[15] to $18·82 ± 4·14$ (carotid–brachial).[32] At follow-up, only one study[15] reported a significant change in PWV where mean experimental PWV declined 0·08 m/s compared to the control group where mean PWV increased 0·37 m/s. Overall, there was significant heterogeneity in response to vitamin D supplementation where five studies[15,16,22,28,30] reported reductions in PWV and seven reported increases in PWV[17,18,21,23,25,27,32] in patients receiving vitamin D treatment (Fig. 3). One study did not provide sufficient data to enable comparison between baseline and follow-up PWV measurements.[24]

Augmentation index

At baseline, all studies reported no significant differences in AI (%) between experimental and control groups except for Whitham *et al.*[30] where control group patients had considerably higher AI than experimental patients [27% *vs* 16%, respectively, $P = 0·001$] (Table 4). In experimental groups, AI (%) ranged from $8·5 ± 1·1$[22] to $78·8 ± 13$[18] and in control groups, the range was from $8·5 ± 1·5$[22] to $80·5 ± 11·4$.[18] At follow-up, three studies reported significant differences in AI between experimental and control groups.[16,20,27] AI was significantly reduced in[16,20] but was increased in[27] (Table 2). Overall, there was significant heterogeneity in response to vitamin D supplementation where four studies reported reductions in AI in patients receiving vitamin D compared to control[16,20–22] and

Table 1. Study details

Study	Design	Cohort	Vitamin D deficient/ insufficient at recruitment	Total population (n)	Follow-up (months)	Dose	Daily dose (equivalent IU/day)	Outcome	Inclusion/Exclusion criteria
Bresklavksy	RCT, DB, PC	T2D	No	47	12	1000 IU/day	1000	AI	E: Unstable angina, MI, CVA, major surgery, hyperthyroidism, creatinine>2·5 mg/dl, elevated liver enzymes
Dong	RCT, OL	African American youths	No	49	4	2000 IU v 400 IU	2000	PWV femoral, radial, distal	I: Normotensive, 14–18 years old, currently not taking medications, can swallow, able to provide blood, not pregnant, not on vitamin D supplements
Dreyer	RCT, DB, PC	CKD 3–4	Yes	29	6	50 000 IU/4 week, 50,000 IU/5 month	1666	PWV	E: Currently on calcium therapy >10·4 mg/dl, pregnant or lactating, hypercalcaemia, microcirculatory dysfunction
Garg	RCT, DB, PC	PCOS	No	32	6	1 × 120 000 IU/month	4000	PWV, AI	E: Currently on vitamin D supplements, active disease, currently taking medications known to interact with vitamin D, pregnant
Gepner 2012	RCT, DB, PC	Postmenopausal women	Yes	110	4	2500 IU/daily	2500	PWV, AI	I: vitamin D between 10 and 60 ng/mL, otherwise healthy, community-dwelling postmenopausal women. E: History of CVD, calcium >10·5 mg/dl, hyperparathyroidism, malignancy, tuberculosis, nephrolithiasis, sarcoidosis, Paget's disease, eGFR<25 ml/min, medications known to interact with vitamin D
Gepner 2015	RCT, DB	Postmenopausal native American women	Yes	98	6	2500 IU v 400 IU daily	2500	AI	I: vitamin D between 10 and 60 ng/ml, no CVD
Hewitt	RCT, DB, PC	HD, CKD5	Yes	60	6	8 × 50 000 IU/weekly, 4 × 50 000 IU/monthly	3611	PWV	I: vitamin D between 10 and 60 ng/mlE: Parathyroid surgery, cinacalcet treatment, hypercalcaemia, bisphosphonate therapy, major surgery
Larsen	RCT, DB, PC	Hypertension	No	112	5	3 × 25 µg daily	3000	PWV, AI	E: SBP>150 mmHg and/or DBP>95 mmHg, pregnancy/lactating, alcohol abuse, hypercalcaemia, atrial fibrillation, NSAID use, glucocorticoids, current vitamin D intake >10 µg, tanning bed use, changes in antihypertensive medications during trial
Marckmann	RCT, DB, PC	CKD	Yes	52	2	40 000 IU/weekly	5700	PWV, AI	I: vitamin D < 50 nmol/lE: current vitamin D intake, hypercalcaemia, hyperphosphataemia, sarcoidosis, malignancy, psychosis, alcohol/drug abuse, pregnancy/lactating, poor language skills, soy allergy, oestrogen use, contraceptive use

(continued)

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Table 1. (continued)

Study	Design	Cohort	Vitamin D deficient/ insufficient at recruitment	Total population (n)	Follow-up (months)	Dose	Daily dose (equivalent IU/day)	Outcome	Inclusion/Exclusion criteria
Martins	RCT, DB, PC	Hypertensive African Americans	Yes	115	3	100 000 IU/monthly	3333	AI	I: serum levels of 25(OH)D 10 and 25 ng/mlE: Poorly controlled BP, CKD, hypercalcaemia, abnormal liver function tests, MI, stroke, congestive heart failure, kidney stones, allergy to oral vitamin D, current immunosuppressive therapy, current steroid therapy and current NSAID
McGreevy	RCT, DB	Older community-dwelling individuals (>65 years)	Yes	102	2	100 000 IU vs 50 000 IU single	1666	PWV, AI	I: serum 25OHD <50 nmol/lE: Currently taking vitamin D supplements, hypercalcaemia, hyperparathyroidism, current malignancy, change in medications during trial
Mose	RCT, DB, PC	HD	No	50	6	75 ug/daily	3000	PWV, AI	E: Malignancy, hypercalcaemia, allergy to vitamin D, inability to give consent (<18 years)
Pilz	RCT, DB, PC	Hypertension	Yes	183	2	2800 IU as oil drops daily	2800	PWV	I: 25(OH)D serum concentration below 30 ng/mlE: Hypercalcaemia, pregnancy/lactating, taking drugs from other studies, acute coronary disease, CVD, eGFR<15 mls/min/1·73 m², SBP between 120 and 160 mmHg, DBP >100 mmHg, taking hypertensive drugs, life expectancy <10 years, receiving chemotherapy or radiotherapy, regular vitamin D supplement intake
Ryu	RCT, DB, PC	T2D	Yes	81	6	2000 IU/day (+100 mg/day Ca)	2000	PWV, AI	I: I: 25(OH)D < 20 ng/mlE: Osteoporosis drugs, insulin use, SB >160 mmHg or DBP >100 mmHg, recent MI, abnormal liver enzymes, alcohol abuse
Stricker	RCT, DB, PC	PAD	Yes	62	1	1 × 100 000 IU	3333	AI	I: serum 25-hydroxyvitamin D level <30 ng/mlE: Acute illness, critical ischaemia, thromboangiitis obliterans, renal insufficiency (Cr <130 μmol/l), recent MI, current oral anticoagulants, liver cirrhosis, malignancy
Whitham 2013	RCT, DB, PC	Older individuals (>70 years) with hypertension (>140 mmHg SBP)	Yes	159	12	4 × 100 000 IU monthly	3333	PWV	I: 25OHD level <30 ng/mlE: DBP >90 mmHg, SBP>180 mmHg, eGFR <40 mls/min/1·73 m², abnormal liver function tests, metastasis malignancy, sarcoidosis, renal calculi, heart failure, left ventricular dysfunction, atrial fibrillation, already on vitamin D supplements

(continued)

eight studies reported increases in AI for patients receiving vitamin D[17,18,23,25–27,29,30] (Fig. 4).

Meta-analysis

Thirteen studies had data that was able to be synthesized into a meta-analysis model.[20–32] The authors of studies that were eligible for meta-analysis but did not report appropriate data for synthesis were contacted directly but did not respond to our communications.[17,18] For the outcome of PWV, vitamin D supplementation produced a nonsignificant reduction in PWV relative to placebo (Table 5, Fig. 3). Similarly for AI, vitamin D supplementation produced a nonsignificant reduction in AI relative to placebo (Table 5, Fig. 4). Additionally, a number of smaller models were constructed to determine the influence of vitamin D dosing, follow-up, age and the sample in which the studies were conducted in. All subanalyses showed nonsignificant reductions in PWV or AI (Table S4), except for an analysis involving two studies of patients with T2D or PCOS (insulin-resistant syndromes) which showed vitamin D to be nonsignificantly related with an increase in PWV (Table S4).

Discussion

This systematic review of 18 randomized controlled trials assessing the effect of vitamin D supplementation on PWV and AI, two measures of vascular compliance, found three studies that reported significant but conflicting differences in AI and only one study that reported a significant decrease in PWV following vitamin D supplementation. Studies that fulfilled our inclusion and exclusion criteria were largely of high quality but were heterogeneous in terms of study design, patient sample, follow-up times, vitamin D regimen and outcome. A meta-analysis demonstrated that vitamin D supplementation produced small, nonsignificant reductions in PWV and AI relative to placebo. In subanalyses, where we attempted to explore possible explanations for the variable response to vitamin D on these outcomes, we found no significant associations in terms of study follow-up time, study sample, sample age or vitamin D dosing. These results are in contrast to a large amount of observational clinical studies which have shown that low vitamin D (defined as <50 nmol/l) is associated with poor measures of arterial stiffness.[34,35] For example, in the NHANES longitudinal studies from 2001 to 2004, the risk of developing peripheral arterial disease, a condition largely characterized by arterial stiffness, was almost twice as high in subjects with vitamin D < 50 nmol/l compared to those with vitamin D > 75 nmol/l (odds ratio 1·82; 95% CI:1·26–2·61).[5]

Preclinical studies have also provided strong evidence that vitamin D is an important factor in maintaining good vascular compliance and a number of possible mechanisms by which vitamin D may influence vascular compliance have been proposed. In particular, *in vitro* studies have suggested that vitamin D deficiency is associated with increased vascular endothelial cell (EC) expression of nuclear factor κβ (NFκβ) and interleukin-6 (IL-6), two important inflammatory mediators.[36] Further, vitamin D may attenuate the adverse effects of advanced glycation

Table 1. (continued)

Study	Design	Cohort	Vitamin D deficient/ insufficient at recruitment	Total population (n)	Follow-up (months)	Dose	Daily dose (equivalent IU/day)	Outcome	Inclusion/Exclusion criteria
Whitham 2015	RCT, DB, PC	CFS	Yes	50	6	3 × 100 000 IU (once every 2 months)	3333	PWV, AI	I: serum 25OHD level <75 nmol/l;E: Osteoporosis, sarcoidosis, renal stones, malignancy, current vitamin D intake, abnormal liver function tests, hypercalcaemia, eGFR < 40 mls/min/1·73 m², no consent given, psychiatric disorders, substance abuse/dependence
Yiu	RCT, DB, PC	T2D	Yes	100	3	5000 IU/daily	5000	PWV	I: serum 25(OH)D concentration <30 ng/mlE: HbA1c >11%, pregnancy, lactation, recent MI, angina, Cr >106 μmol/l, liver failure, cancer, uncontrolled hypertension, diabetes complications

E, Exclusion criteria; I, inclusion criteria; RCT, randomized controlled trial; DB, double blind; SB, single blind; OL, open label; CKD, chronic kidney disease; HD, haemodialysis; PC, placebo-controlled; PCS, prospective cohort study; CFS, chronic fatigue syndrome; SBP, systolic blood pressure; PAD, peripheral arterial disease; PCOS, polycystic ovarian syndrome; T2D, type 2 diabetes mellitus; AI, augmentation index; PWV, pulse wave velocity; MI, myocardial infarction.

Table 2. Patient demographics and clinical characteristics

Study	n Exp.	n Cont.	Age (years) Exp.	Age (years) Cont.	Age P	Male sex [n(%)] Exp.	Male sex [n(%)] Cont.	Male sex P	BMI (kg/m²) Exp.	BMI (kg/m²) Cont.	BMI P
Bresklavksy	24	23	66·8 ± 9·2	65·8 ± 9·7	0·716	11 (45·8%)	11 (47·8)	0·562	27·9 ± 5·2	30·6 ± 5·1	0·073
Dong	23	21	16·5 ± 1·4	16·3 ± 1·1	0·95	10 (43%)	15 (71)	0·08	n/r	n/r	n/r
Dreyer	20	18	45·8 ± 10·0	48·8 ± 12·2	0·39	14 (60·9)	14 (73·7)	0·22	30·4 ± 7·1	29·2 ± 3·4	0·51
Garg	15	17	22·0 ± 4·61	22·8 ± 4·56	0·64	n/a	n/a	n/a	26·8 ± 4·56	26·7 ± 6·11	0·96
Gepner 2012	57	57	64·1 ± 3·0	63·6 ± 3·1	0·419	n/a	n/a	n/a	27·1 ± 4·7	25·3 ± 5·1	0·002
Gepner 2015	49	49	60·7 ± 7·7	61·8 ± 7·0	n/r	53	43	n/r	32·4 ± 6·4	33·3 ± 7·3	n/r
Hewitt	30	30	60 (53–71)	67 (54–72)	n/r	n/r	n/r	0·94	26·6 ± 6·4	31·3 ± 9·5	n/r
Larsen	55	57	60 ± 12	61 ± 9	0·78	17 (30)	18 (32)	n/r	27·7 ± 4·2	28·3 ± 3·7	0·41
Marckmann	26	26	71 (62–78)	68 (59–76)	n/r	19	20	n/r	25·9 (22·1–29·7)	24·6 (22·0–27·3)	n/r
Martins	60	55	n/r	n/r	n/r	41 (63·1)	38 (58·5)	0·16	n/r	n/r	n/r
Mc Greevy	51	51	79·3 ± 7	80·5 ± 6·6	0·37	28	26	0·556	26·6 ± 6·4	26·9 ± 9·5	0·85
Mose	25	25	68 ± 9	67 ± 13	0·794	17 (68)	15 (60)	0·777	24 ± 4·5	23·8 ± 4·4	0·856
Pilz	100	100	60·5 ± 10·9	59·7 ± 11·4	0·607	54	52	n/r	30·4 ± 4·4	30·4 ± 6·2	0·967
Ryu	40	41	54·5 ± 7·4	56·7 ± 7·9	0·203	19 (61)	19 (61)	0·94	24·4 ± 5·0	25·3 ± 3·4	0·334
Stricker	31	31	72·9 ± 8·7	74·8 ± 14·6	0·5	40 (50)	42 (53)	n/r	n/r	n/r	n/r
Whitham 2013	80	79	76·9 ± 4·8	76·7 ± 4·5	n/r	7 (28)	5 (20)	n/r	28·5 ± 5	27·9 ± 4·5	n/r
Whitham 2015	25	25	48·1 ± 12	50·7 ± 13·1	0·47	27 (54)	23 (46)	0·51	28·8 ± 7·9	29·8 ± 5·4	0·6
Yiu	50	50	65·8 ± 7·3	64·9 ± 8·9	0·58	27 (54)	23 (46)	0·42	25·8 ± 4·3	25·1 ± 3·4	0·34

Study	T2D [n(%)] Exp.	T2D [n(%)] Cont.	T2D P	Smoker [n(%)] Exp.	Smoker [n(%)] Cont.	Smoker P	CVD [n(%)] Exp.	CVD [n(%)] Cont.	CVD P	Hypertension [n(%)] Exp.	Hypertension [n(%)] Cont.	Hypertension P	Dyslipidaemia [n(%)] Exp.	Dyslipidaemia [n(%)] Cont.	Dyslipidaemia P
Bresklavksy	n/r	n/r	n/r	6 (25)	3 (13)	0·461	n/r	n/r	n/r	19 (79·2)	20 (87·0)	0·701	20 (83·3)	20 (87·0)	1
Dong	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r
Dreyer	n/r	n/r	n/r	1 (5)	2 (11·1)	n/r	n/r	n/r	n/r	16 (80)	12 (66·7)	0·33	9 (45)	7 (38·9)	0·84
Garg	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r
Gepner 2012	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r
Gepner 2015	10 (20)	13 (27)	n/r	n/r	n/r	n/r	n/r	n/r	n/r	27 (55)	27 (55)	n/r	23 (47)	21 (43)	n/r
Hewitt	15	18	n/r	13	15	n/r	17	19	n/r	n/r	n/r	n/r	n/r	n/r	n/r
Larsen	4 (7)	5 (9)	0·77	4 (7)	5 (9)	0·77	n/r	n/r	n/r	47 (84)	48 (84)	0·85	17 (30)	19 (33)	0·78
Marckmann	8 (31)	10 (38)	n/r	n/r	n/r	n/r	10 (28)	13 (50)	n/r	23 (88)	18 (69)	n/r	n/r	n/r	n/r
Martins	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r
Mc Greevy	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r
Mose	2 (8)	5 (20)	0·384	1 (4)	5 (20)	0·22	8	5	0·39	17 (68)	17 (68)	0·914	n/r	n/r	n/r
Pilz	32	41	0·186	19	14	0·85	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r
Ryu	100	100	n/a	17 (42·5)	15 (36·6)	0·341	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r
Stricker	10 (32)	7 (23)	0·57	n/r	n/r	n/r	n/r	n/r	n/r	24 (77)	23 (74)	0·77	n/r	n/r	n/r
Whitham 2013	11 (14)	11 (14)	n/r	8n	14n	n/r	n/r	n/r	n/r	41 (51)	50 (63)	n/r	41 (51)	46 (58)	n/r
Whitham 2015	3 (12)	1 (4)	0·61	4 (16)	6 (24)	0·73	1 (4)	3 (12)	n/r	4 (16)	5 (20)	0·71	2 (4)	4 (8)	0·67
Yiu	100	100	n/a	15 (30)	13 (26)	0·66	23 (46)	17 (34)	0·22	40 (80)	42 (84)	0·6	40 (80)	39 (78)	0·81

n.b All values in mean ± SD or mean(SE) or median(interquartile range). exp, Experimental (vitamin D) group; cont, Control (placebo) group; n/a, not applicable; n/r, not reported.
exp, Experimental (vitamin D) group; cont, Control (placebo) group; n/a, not applicable; n/r, not reported.

Table 3. Outcome of PWV from Vitamin D supplementation

	PWV site	Baseline measurement (m/s)			Follow-up measurement (m/s)		Mean difference (within group)		Mean difference (between group)	
		Experimental	Control	P	Experimental	Control	Experimental	Control	P	
Dong	Femoral	5·41 ± 0·73	5·38 ± 0·53	n/r	5·33 ± 0·79	5·71 ± .075	−0·08	0·37	−0·45	0·019
	Radial	7·83 ± 1·14	7·77 ± 1·64	n/r	7·81 ± 0·98	7·92 ± 0·89	−0·02	0·15	−0·17	0·93
	Distal	6·75 ± 0·64	6·87 ± 0·64	n/r	6·71 ± 0·63	7·22 ± 0·79	−0·04	0·25	−0·29	0·46
Dreyer		8·5 ± 1·1	8·5 ± 1·5	0·66	8·4 ± 1·3	8·5 ± 1·2	−0·1	0	−0·1	0·78
Garg		5·6 ± 1·3	6·5 ± 1·25	0·61	6·2 ± 1·32	6·3 ± 1·04	0·6	−0·2	0·8	0·16
Gepner 2012		7·8 ± 0·9	8·0 ± 1·4	0·426	n/r	n/r	0·05	0	0·05	0·625
Hewitt		n/r	n/r	n/r	9·3 ± 3·3	10·5 ± 2·8	n/r	n/r	n/a	0·76
Larsen		8·5 ± 2·3	8·7 ± 2·1	n/r	9·0 ± 2·5	9·0 ± 2·5	0·4	0·3	0·1	0·66
Marckmann		10·4 ± 4·2	10·7 ± 6·8	n/r	8·9 ± 3·6	8·5 ± 3·5	− 1·5	−1·2	−0·3	0·750
Mc Greevy	PWV	12·55 ± 5·36	11·24 ± 2·21	n/r	11·1 ± 2·3	11·1 ± 2·3	−1·45	−0·14	−1·31	0·097
Mc Greevy	PWV (adapted)	11·94 ± 4·75	10·1 ± 2·77	n/r	10·61 ± 1·83	10·22 ± 2·55	−1·33	0·12	−1·45	0·071
Mose		9·7 ± 2·5	10 ± 2	n/r	10·5 ± 4	10·1 ± 2·5	0·8	0·1	0·7	0·269
Pilz		8·41 ± 1·97	8·26 ± 2·06	0·669	8·48 ± 2·22	8·64 ± 2·42	0·07	0·38	−0·31	0·302
Ryu		15·79 ± 2·56	15·74 ± 2·88	0·934	n/r	n/r	−0·16	−0·6	0·44	0·348
Whitham 2013		8·8 ± 1·2	8·7 ± 1·2	n/r	8·8 ± 1·4	9·0 ± 1·4	0·0	0·2	−0·2	0·40
Whitham 2015		7·3 ± 2·6	8·3 ± 1·9	0·13	6·9 ± 2·4	8·1 ± 1·4	−0·4	−0·2	−0·2	0·93
Yiu	Heart–Carotid	9·25 ± 2·67	10·06 ± 3·4	0·17	9·57	9·74	0·32	−0·32	0·64	0·75
	Heart–Ankle	10·71 ± 2·52	11·16 ± 1·45	0·27	11·25	11·11	0·54	−0·05	0·59	0·28
	*Brachial–Ankle	18·97 ± 3·38	18·82 ± 4·14	0·85	18·92	18·7	−0·05	−0·12	0·07	0·80

*Data used for meta-analysis.
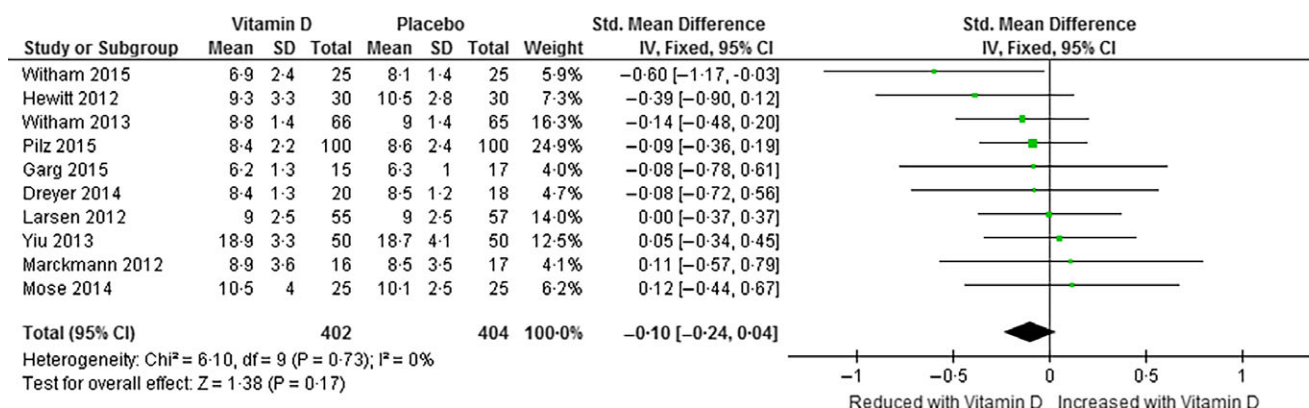
Fig. 3 Forest plot of mean vitamin D- and placebo-treated group data for the outcome of PWV.

end-products on EC which may precipitate dysfunction.[37] Another proposed mechanism from *in vivo* studies is vascular smooth muscle cell (VSMC) modulation, where vitamin D analogues have been shown to upregulate endothelin gene (which in turn can influence the expression of the powerful vasodilator nitric oxide) and further downregulate oxytocin receptor gene in VSMC, an effect which favours vessel relaxation.[38] Other studies have shown that exogenous supplementation of vitamin D resulted in decreased EC proliferation and that EC stress upregulates vitamin D receptor expression on EC creating an autocrine/paracrine role for vitamin D with the potential to influence or modulate EC adhesion and VSMC migration and proliferation.[39] These results indicate that vitamin D is, at the molecular/cellular level, critical to the proper working of the vasculature. Taken together, it is these observational and preclinical data that have provided the evidence to suggest that vitamin D supplementation may improve measures of arterial stiffness. However, the interventional clinical studies surveyed in this review have not demonstrated that vitamin D supplementation results in improved vascular compliance.

This review identified 18 randomized interventional trials that sought to determine whether vitamin D supplementation (using cholecalciferol/vitamin D_3) would improve PWV or AI. There were significant sources of heterogeneity and study quality between these studies that may explain the apparent lack of, and inconsistent, effects across this literature. Principally, the dosing regimen employed varied substantially and thus may account for the most heterogeneity in effect. Only two studies failed to

Table 4. Outcome of AI from Vitamin D supplementation

Study	Baseline (%)			Follow-up (%)			Mean difference (within groups)		Mean difference (between groups)
	Experimental	Control	P	Experimental	Control	P	Experimental	Control	P
Bresklavksy	32·9 ± 11·9	29·5 ± 10·9	0·314	25·9 ± 9·4	27·2 ± 9·3		−7	−2·3	−4·7	0·01
Dreyer	8·5 ± 1·1	8·5 ± 1·5	0·66	8·4 ± 1·3	8·5 ± 1·2		−0·1	0	−0·1	0·78
Garg	11·9 ± 10·72	12·1 ± 7·73	0·98	10·6 ± 10·5	11·4		−1·3	−1·5	0·2	0·78
Gepner 2012	n/r	n/r	n/r	n/r	n/r		2·7	0·9	1·8	0·096
Larsen	26 ± 7	26 ± 9	n/r	25 ± 9	26 ± 8		−1	0	−1	0·37
Marckmann	28 (22–31)	26 (18–30)	n/r	n/r	n/r		−1·5	−2	0·5	n/r
Martins	28·2 ± 11·2	31 ± 12	0·1824	27·6 ± 11	29·3 ± 11		−0·6	−1·7	1·1	n/r
Mc Greevy	29·4 ± 6·9	28·5 ± 7·2	n/r	25·6 ± 1·2	28·4 ± 0·9		−3·8	−0·1	−3·7	0·033
Mose	22·1 ± 9·7	26·4 ± 11·5	n/r	24·6 ± 12·7	22·2 ± 11·2		2·5	−4·2	6·7	0·013
Ryu	78·8 ± 13	80·5 ± 11·4	0·595	n/r	n/r		−2·2	−4·3	2·1	0·399
Stricker	38·6 ± 7·3	39·5 ± 7·1	0·77	38·5 ± 8·7	38·6 ± 7·2		−0·1	−0·9	0·8	n/r
Whitham 2015	16 ± 13	27 ± 10	0·001	15 ± 12	25 ± 9		−1	−2	1	0·16
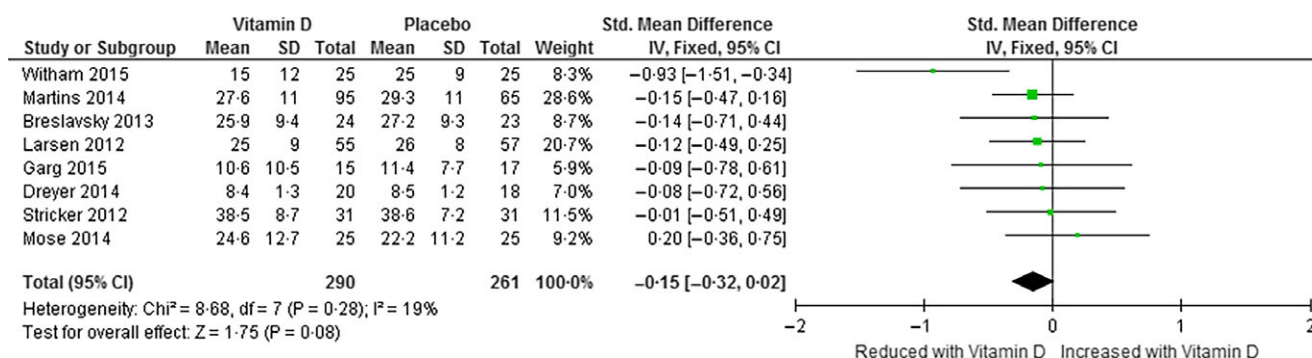
Fig. 4 Forest plot of mean vitamin D- and placebo-treated group data for the outcome of AI.

Table 5. Summary of meta-analysis for the outcome of PWV and AI

Analysis	# Studies	# Participants	SMD [95% CI]	P	I^2	Effects model
Pulse Wave Velocity	10	806	−0·10 [−0·24, 0·04]	0·17	0	Fixed
Augmentation Index	8	551	−0·15 [−0·32, 0·02]	0·08	19	Fixed

SMD, standardized mean difference; 95% CI, 95% confidence interval; I^2, inconsistency percentage.

achieve mean vitamin D adequacy in their experimental group.[16,20] Participants in these two studies were severely vitamin D deficient, which may explain their failure to achieve adequate vitamin D status during the intervention period. Alternatively, this could be due in part to the relatively low dose employed (1000 IU/day[20]) or the fact vitamin D was given as a single dose at the start of their trial.[16] Indeed, the optimal vitamin D dosing protocol in terms of concentration and frequency is unclear.[40] This is a consistent technical problem in study design, as many interventional studies that are grounded in epidemiological evidence suggesting low vitamin D is associated

with an outcome, show no effect for vitamin D supplementation.[41] However, attempts to overcome this with high-dose vitamin D supplementation may be harmful.[42] Overall, vitamin D supplementation was largely successful in achieving adequate mean vitamin D levels; however, there was no significant difference in outcome in meta-analyses involving only studies with high-dose vitamin D or a low-dose vitamin D. However, for studies that reported decreases in PWV[15,16,22,28,30] or AI[16,20,21] five of these studies employed a vitamin D less than 3000 IU/day[15,16,20,22,28] and four of these studies had follow-up times >6 months.[15,16,21,28] Further evidence of a dose-dependent effect

is supported by the fact that the studies reporting an increase in PWV in experimental groups relative to controls[18,21,23,25,27,32] all but one used a vitamin D dose less than 3000 IU/day[18]; however, this study had the longest follow-up time suggesting that the duration of vitamin D supplementation may be important in influencing the outcome. Indeed, orthodox theory is that studies conducted over relatively short time frames (e.g. 1–3 months) may not be sufficient to witness discernible and significant differences in outcome. However, in subanalyses comparing the outcome in studies with a shorter or longer follow-up time, there were no significant differences in vitamin D-treated groups compared to placebos. In many instances, follow-up vitamin D was not reported making it difficult to comment on the overall efficacy of vitamin D supplementation.[15,17,21,22,24,25,29,31] Genetic differences in the vitamin D receptor between the samples (an aspect not explored in any study) may potentially explain much of the responsiveness to vitamin D supplementation and may help guide dosing protocols. In considering the diversity of the samples that make up the literature aggregated in this review, there is a high likelihood that there are significant genetic differences in vitamin D and receptor biology.[43] Further, recent evidence is emerging to suggest that 1,25-dihydroxyvitamin D, the active metabolite of 25OHD, may be more relevant to cardiovascular disease where it has a positive relationship to the risk of hypertension in contrast to the negative relationship of 25OHD.[44] 1,25-dihydroxyvitamin D is associated with a higher urinary calcium possibly indicating increased calcium absorption that may promote vascular calcification and increase arterial stiffness. Importantly, though, the activity or concentration of enzymes involved in the synthesis pathway of the active vitamin D metabolite were not quantified in this previous study[44] and this may offer another explanation for the contradictory relationships found between 25OHD and 1,25-dihydroxyvitamin D. The age and body composition of the samples considered in this review were heterogeneous between the samples. This means in the meta-analysis we are aggregating samples of older and younger, overweight and normal weight samples which may affect the mechanism of vitamin D. Vitamin D is a fat soluble steroid hormone and is known to become sequestered in adipose tissue as adiposity increases, which may limit vitamin D receptor sensitivity.[45] Therefore, in the light of these aspects, it may be the case that vitamin D dosing may need to be better targeted taking into account the person's adiposity and genetic variants in the vitamin D receptor which have previously been described for the risk of diabetes.[46]

Despite a many number of observational studies showing associations between low vitamin D concentrations and a wide variety of diseases and outcome, an equally large number of randomized interventional trials have not confirmed the hypothesis that raising vitamin D concentrations can modify the occurrence or clinical course of these disorders.[47] Hence, associations between vitamin D and health disorders reported by investigators of observational studies are not causal. Low vitamin D could well be the result of inflammatory processes involved in the occurrence and progression of disease. Evidence in critically ill patients with acute health conditions characterized by severe inflammation support this, as vitamin D concentrations fall substantially during acute health events.[48] In the light of these aspects, vitamin D as a stand-alone measure may be insufficient and that it may need to be combined with an adjunctive therapy in order to improve arterial stiffness and lower CVD risk. Indeed, as a demonstration of its adjunctive capacity, a study conducted in obese older adults showed 5-year gains in fat tissue were smaller in people with higher concentrations of 25OHD at baseline and had higher levels of physical activity; suggesting that physical activity may be required to enhance the effect of vitamin D.[49]

The current review has some limitations. Firstly, the authors did not have access to primary data and thus were limited in the statistical analyses performed. Secondly, data extraction was performed by a single reviewer only. Thirdly, we considered PWV and AI measurements based on assessments of different vascular regions (e.g. brachial–ankle and carotid–radial) using different devices. This is significant as some sites are better predictors of CVD outcome than others, and indeed, more standardized measurement protocols are required.[50] Finally, during screening, we excluded a number of records based on the fact that the record existed only in conference proceedings/abstract form and has not been published as a full-text manuscript. This means that there may be a significant amount of data, not yet publically available, that could potentially contribute to the conclusions reached in this review. However, some data were obtained directly from authors although as these results are not yet published, we cannot guarantee these results were subject to full peer review and thus it was prudent to not include them in our critique of the literature.

In conclusion, this review sought to clarify the literature surrounding the use of vitamin D supplementation on the outcome of arterial stiffness in randomized controlled trials. We found no conclusive evidence to suggest that vitamin D supplementation is beneficial despite overwhelming observational evidence suggesting low vitamin D is a risk factor for increased arterial stiffness. Indeed, there was significant heterogeneity in the effect of vitamin D supplementation. This may be related to study design in terms of dosing protocol and the samples in which these studies were conducted. Due to its pleiotropic effects, low vitamin D is associated with a number of diseases, but few casual associations have been established.[51] Therefore, large, robust and well-designed RCTs and prospective longitudinal studies are required to determine the potential casual nature of vitamin D on arterial compliance and CVD risk independent of known risk factors.

Author contributions

expert opinion on vitamin D biology, contributed to manuscript development and reviewed the final draft.

Declaration

The authors declare no conflicts of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site.

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SUPPLEMENTARY MATERIALS

MANUSCRIPT CEN-2015-000995

RODRIGUEZ ET AL. 2015 "Effect of Vitamin D Supplementation on Measures of Arterial Stiffness: A Systematic Review of Randomised Controlled Trials"

Supplementary Table 1. PWV measurement details

Study	Region	Device
Bresklavksy	Carotid-radial (crPWV)	SphygmoCor CPV system; Atcor Medical, Sydney, Australia
Dong	Carotid-radial, carotid-femoral, carotid-dorsalis-pedis (foot)	Millar Instruments, Houston, TX
Dreyer	Aortic	Vicorder system (Skidmore Medical)
Garg	Carotid-femoral (cfPWV)	SphygmoCor CPV system; Atcor Medical, Sydney, Australia
Gepner 2012	Carotid-femoral	SphygmoCor CPV system; Atcor Medical, Sydney, Australia
Gepner 2015	n/r	SphygmoCor CPV system; Atcor Medical, Sydney, Australia
Hewitt	n/r	SphygmoCor CPV system; Atcor Medical, Sydney, Australia
Larsen	n/r	SphygmoCor CPV system; Atcor Medical, Sydney, Australia
Marckmann	n/r	Millar Instruments, Houston, TX
Martins	Carotid-radial	SphygmoCor CPV system; Atcor Medical, Sydney, Australia
Mc Greevy	Carotid-femoral	Vicorder system (Skidmore Medical)
Mose	Carotid-femoral	SphygmoCor CPV system; Atcor Medical, Sydney, Australia
Pilz	Carotid-femoral	SphygmoCor CPV system; Atcor Medical, Sydney, Australia
Ryu	Brachial-ankle (baPWV)	Waveform Analyser Model VP-2000, Colin, Komaki, Japan
Stricker	Carotid-radial	SPC-301, Millar Instruments, Houston, TX, USA
Whitham 2013	Carotid-radial	SphygmoCor CPV system; Atcor Medical, Sydney, Australia
Whitham 2015	Carotid-femoral	SphygmoCor CPV system; Atcor Medical, Sydney, Australia
Yiu	Heart-carotid (hcPWV), heart-ankle (haPWV), brachial-ankle	VP-2000 System (Colin Corp., USA)

Supplementary Table 2. Study quality assessment using items adapted from the Jadad scale (14)

Study	Item													Score
	1	2	3	4	5	6	7	8	9	10	11	12	13	
Breslavsky	*	*		*	*	*	*		*	*	*		*	10
Dong	*	*	*	*	*	*	*		*	*	*		*	11
Dreyer	*	*	*	*	*	*	*		*	*	*		*	11
Garg	*	*	*	*	*	*	*		*	*	*	*	*	12
Gepner 2012	*	*	*	*	*	*	*		*	*	*		*	11
Gepner 2015	*	*		*	*		*		*	*	*		*	9
Hewitt	*	*		*	*		*		*	*	*		*	9
Larsen	*	*	*	*	*		*		*	*	*		*	10
Marckmann	*	*	*	*	*		*		*		*		*	9
Martins	*	*	*	*	*	*	*		*	*	*		*	11
McGreevy	*	*	*	*	*	*	*		*		*		*	10
Mose	*	*	*	*	*	*	*		*	*	*		*	11
Pilz	*	*	*	*	*	*	*		*	*	*		*	11
Ryu	*	*	*	*	*	*	*		*	*	*		*	11
Stricker	*	*	*	*	*	*	*		*		*		*	10
Witham 2013	*	*	*	*	*	*	*		*	*	*		*	11
Witham 2015	*	*	*	*	*	*	*		*	*	*		*	11
Yiu	*	*	*	*	*	*	*		*	*	*		*	11

Supplementary Table 3. Vitamin D (ng/mL) Supplementation details

Study	Blood medium	Method	Baseline (ng/mL)			Follow Up (ng/mL)		
			Experimental	Control	p	Experimental	Control	p
Bresklavksy	Plasma	Competitive protein-binding assay	12.91±10.69	10.79±6.57	0.431	17.6±11.5	14.0±5.9	0.299
Dong	Plasma	IDS 25-hydroxyvitamin D EIA kit (Immunodiagnostics Systems)	33.1±8.7	34.0±10.6	0.76	n/r	n/r	n/r
Dreyer	Serum	Quantitative ultra-performance liquid chromatography tandem mass spectrometry assay	n/r	n/r	n/r	n/r	n/r	n/r
Garg	Serum	Direct competitive chemiluminescence immunoassay (DiaSorin Liaison, Stillwater, MN, USA).	7.7±6.05	6.8±2.46	0.96	31.5±13.88	6.7±2.31	<0.001
Gepner 2012	Serum	High performance liquid chromoatography	30.3±10.7	32.3±10.5	0.353	n/r	n/r	n/r
Gepner 2015	Serum	n/r	27.2±9.3	25.1±10.1	n/r	42.7±11.6	30.1±7.5	n/r
Hewitt	Serum	Liaison assay (DiaSorin Inc, Stillwater, MN)	18±5	18±10	n/r	n/r	n/r	n/r
Larsen	Plasma	Chemiluminescence immunoassay (DiaSorin, Saluggia, Italy)	23±9	23±12	0.74	n/r	n/r	n/r
Marckmann	Plasma	Mass spectrometry (LCMSMS1, Applied Biosystems, Dionex, Sunnyvale, California, US)	9.5 (6.9-16.6)	13.3 (9.5-17.7)	n/r	59.3±15.1	8.9±5.1	<0.001
Martins	Serum	n/r	17±5.2	16.5±5	0.4488	34.5±7.1	17.2±6.4	n/r
Mc Greevy	Serum	Liquid chromatography/mass spectrometry	11.1±4.7	10.9±5.4	n/r	19.4±6.7	16.5±5.4	0.022
Mose	Plasma	Chemiluminescence immunoassay (DiaSorin, Saluggia, Italy)	11.2(8-19.2)	11.2(8.0-27.6)	n/r	33.7(26-50.1)	12(8.8-20)	<0.001
Pilz	Serum	IDS 25-hydroxyvitamin D EIA kit (Immunodiagnostics Systems)	22.0±5.7	20.5±5.7	0.055	36.2±7.3	23.6±8.9	<0.001
Ryu	Serum	Direct competitive chemiluminescence immunoassay (DiaSorin Liaison, Stillwater, MN, USA).	12.3±3.0	10.7±2.6	0.188	35.4±8.5	18.4±7.3	<0.001
Stricker	Serum	Radioimmunoassay (RIA) double antibody assay (DiaSorin, Saluggia, Italy)	16.3±6.7	17.0±5.5	0.63	n/r	n/r	n/r
Whitham 2013	Serum	n/r	18±6	18±6	n/r	n/r	n/r	n/r
Whitham 2015	Serum	IDS 25-hydroxyvitamin D EIA kit (Immunodiagnostics Systems)	17.6±6	19.2±8	0.44	25.6±8.4	17.6±8.8	0.001
Yiu	n/r	IDS 25-hydroxyvitamin D EIA kit (Immunodiagnostics Systems)	21.1±4.4	21.9±4.1	0.4	58.6	23.8	<0.001

[Type here]

Supplementary Table 4. Summary of sub-analyses for the outcome of PWV and AI

Analysis	Study type	# studies	n	SMD [95%CI]	p	I²	Effects model
	Follow-up >3months	4	242	-0.12 [-0.37, 0.13]	0.36	0	Fixed
	Vitamin D dose ≥3000IU/day	6	404	-0.11 [-0.30, 0.09]	0.28	12	Fixed
	Vitamin D dose <3000IU/day	2	238	-0.09 [-0.34, 0.17]	0.51	0	Fixed
PWV	CKD	3	148	-0.14 [-0.46, 0.19]	0.41	0	Fixed
	Hypertension	2	312	-0.06 [-0.28, 0.17]	0.62	0	Fixed
	T2D and PCOS	2	132	0.02 [-0.32, 0.36]	0.91	0	Fixed
	Mean age >55 years	5	522	-0.06 [-0.23, 0.12]	0.53	0	Fixed
	Follow-up >3months	3	272	-0.33 [-0.80, 0.15]	0.18	69	Random
	Follow-up ≤3months	5	401	-0.09 [-0.29, 0.11]	0.36	0	Fixed
	Vitamin D dose ≥3000IU/day	6	466	-0.16 [-0.34, 0.03]	0.09	42	Fixed
AI	Hypertension	2	272	-0.14 [-0.38, 0.10]	0.26	0	Fixed
	T2D and PCOS	2	79	-0.12 [-0.56, 0.33]	0.61	0	Fixed
	Mean age >55 years	4	271	-0.04 [-0.28, 0.20]	0.75	0	Fixed
	Mean age ≤55 years	2	82	-0.53 [-1.35, 0.30]	0.21	70	Random
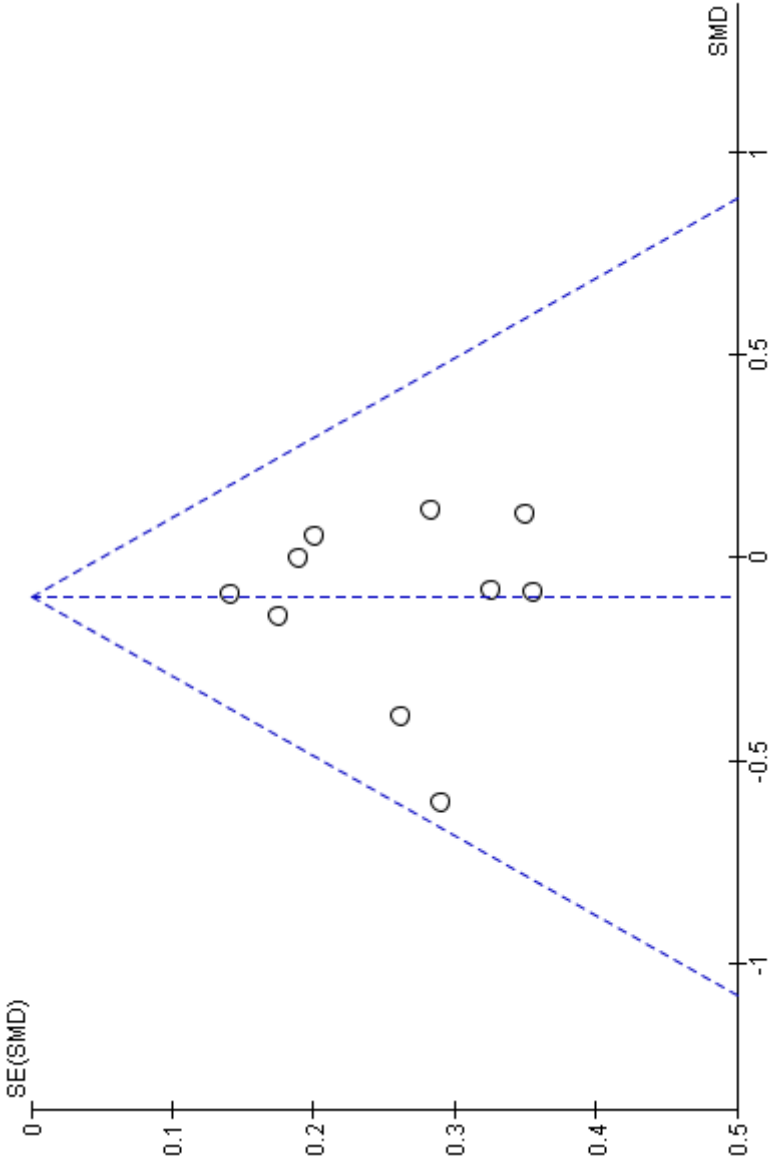
Supplementary Figure 1. Funnel plot for the outcome of PWV

Note: The effect size (difference in mean PWV between cases (those receiving vitamin D and control (those receiving placebo groups) is plotted on the horizontal axis against the standard error for the effect estimate. Standard error is used on the vertical axis instead of population size because the statistical power of a trial is determined by many factors and not solely sample size. For example, a study with 100,000 participants and 10 events is less likely to show a statistically significant intervention effect than a study with 1,000 participants and 100 events. The standard error provides a summary for these other factors. Plotting the standard error on the vertical axis places the larger, or most powerful, studies towards the top of the plot. Further, with this strategy, a simple triangular region can be plotted, within which 95% of studies would be expected to lie in the absence of both biases and heterogeneity. Overall, a symmetrical plot indicates minimal publication bias.

[Type here]

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Supplementary Figure 1. Funnel plot for the outcome of AI

Modified Jadad Scale Questionnaire

1. Was the study described as randomised?

2. Was the study described as double-blind?

3. Was a description of withdrawals/drop-out provided?

4. Were the objectives of the study clearly defined?

5. Were outcome measures clearly defined?

6. Was appropriate methodology employed to assess the outcomes?

7. Was a clear description of the inclusion/exclusion criteria provided?

8. Was the sample size justified (i.e. were power calculations performed)

9. Was there a clear description of the intervention?

10. Was this intervention appropriate and justified?

11. Was there at least one control (or comparison group) group?

12. Were adverse events or other safety data reported?

13. Were statistical methods employed robust and appropriate?

Detailed literature search protocol

(((((((((((("vitamin d"[Title/Abstract]) OR "vit d"[Title/Abstract]) OR "calcitriol"[Title/Abstract]) OR "cholecalciferol"[Title/Abstract]) OR "ergocalciferol"[Title/Abstract]) OR "25 hydroxyvitamin d"[Title/Abstract]) OR "1,25 hydroxyvitamin d"[Title/Abstract]) OR "1,25 oh d"[Title/Abstract]) OR "25 oh d"[Title/Abstract]) OR "vitamin d2"[Title/Abstract]) OR "vitamin d3"[Title/Abstract]) OR "calcidiol"[Title/Abstract]) OR "calciferol"[Title/Abstract] AND ((((((((((("peripheral vascular disease"[Title/Abstract]) OR "peripheral arterial disease"[Title/Abstract]) OR "vascular disease"[Title/Abstract]) OR "arterial disease"[Title/Abstract]) OR "arterial stiffness"[Title/Abstract]) OR "vascular stiffness"[Title/Abstract]) OR "claudication"[Title/Abstract]) OR "pulse wave velocity"[Title/Abstract]) OR "pwv"[Title/Abstract]) OR "augmentation index"[Title/Abstract]) OR "limb ischemia"[Title/Abstract]) OR "limb ischaemia"[Title/Abstract]

Chapter 4.3: Systematic Review

Effects of vitamin D supplementation on inflammatory markers in heart failure: a systematic review and meta-analysis of randomised controlled trials

Rodríguez AJ, Aya M Mousa, David Scott, Peter R. Ebeling & Barbora de Courten

SCIENTIFIC REP🞓RTS

Effects of vitamin D supplementation on inflammatory markers in heart failure: a systematic review and meta-analysis of randomized controlled trials

Alexander J. Rodriguez [1], Aya Mousa[2], Peter R. Ebeling[1], David Scott [1] & Barbora de Courten[2]

Vitamin D is reported to have anti-inflammatory properties; however the effects of vitamin D supplementation on inflammation in patients with heart failure (HF) have not been established. We performed a systematic review and meta-analysis examining effects of vitamin D supplementation on inflammatory markers in patients with HF. MEDLINE, CINAHL, EMBASE, All EBM, and Clinical Trials registries were systematically searched for RCTs from inception to 25 January 2017. Two independent reviewers screened all full text articles (no date or language limits) for RCTs reporting effects of vitamin D supplementation (any form, route, duration, and co-supplementation) compared with placebo or usual care on inflammatory markers in patients with heart failure. Two reviewers assessed risk of bias and quality using the grading of recommendations, assessment, development, and evaluation approach. Seven studies met inclusion criteria and six had data available for pooling (n = 1012). In meta-analyses, vitamin D-supplemented groups had lower concentrations of tumor necrosis factor-alpha (TNF-α) at follow-up compared with controls (n = 380; p = 0.04). There were no differences in C-reactive protein (n = 231), interleukin (IL)-10 (n = 247) or IL-6 (n = 154) between vitamin D and control groups (all p > 0.05). Our findings suggest that vitamin D supplementation may have specific, but modest effects on inflammatory markers in HF.

Heart failure (HF) is a complex and increasingly common condition affecting 26 million people worldwide[1]. HF is associated with morbidity, loss of physical function, and a cascade of neuro-hormonal and peripheral muscle effects[2]. Although the pathophysiology of HF is not fully understood, increasing evidence suggests that the development and clinical course of HF is underscored by an inflammatory milieu including pro- and anti-inflammatory cytokines, adhesion molecules, and reactive oxygen species[3]. Therefore, strategies to reduce inflammation in patients with HF may reduce symptoms and improve overall health outcomes for these patients.

Vitamin D is reported to have anti-inflammatory and immune-modulating properties[4], and a recently published randomized trial reported that vitamin D supplementation improved left ventricular structure and function in patients with HF[5]. A potential role for vitamin D in HF is supported by the widespread distribution of the vitamin D receptor (VDR) and metabolizing enzymes throughout the cardiovascular system, including in cardiac myocytes[6]. Absence of the VDR has resulted in cardiac remodeling and subsequent myocardial hypertrophy in

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1

Total Hits (n=9,762)

| Records identified through **OVID MEDLINE** (n=6,535) & **MEDLINE in Process** (n=760) | Records identified through **EBM Reviews** (n=617) | Records identified through **EMBASE** (n=974) | Records identified through **CINAHL** (n=876) |

Number of duplicates removed (n= 870)

Articles screened by title and abstract (n=8,892) → Published articles excluded (n= 8,688); Grey literature identified and excluded (n= 194)

Additional grey literature: Clinical trials registries (n=4)

Full text articles assessed for eligibility (n=10) → **Full text articles excluded (n=3):**
- Conference or meeting abstract I (n=1)
- No inflammatory markers measured (n=2)

Articles included in data extraction and quality appraisal (n=7)

Studies included in qualitative synthesis (n= 7) → **Articles excluded from meta-analysis (n=1):**
- Unable to obtain relevant data from authors (n=1)

Studies included in quantitative synthesis (pooled in meta-analysis) (n=6)

(Identification / Screening / Eligibility / Extraction / Analysis)

Figure 1. CONSORT diagram of the screening and selection process.

mice[7]. Human epidemiological studies report that vitamin D deficiency [25-hydroxyvitamin D (25(OH)D) concentrations < 50 nmol/l] is common in individuals with HF and has been associated with reduced left ventricular ejection fraction (LVEF), increased natriuretic peptides, and increased mortality[8,9]. Increased concentrations of inflammatory markers in HF patients have also been associated with these same outcomes[10]. In contrast, vitamin D supplementation was recently shown to improve LVEF and reverse LV remodeling[5], improve strength and balance, and reduce the risk of falls in patients with HF[11]. Use of vitamin D supplements may improve HF symptoms and outcomes via an anti-inflammatory pathway. However, the effect of vitamin D supplementation on inflammatory markers in patients with HF has not been established. We aimed to address this knowledge gap by performing a comprehensive systematic review and meta-analysis of all randomized controlled trials (RCTs) examining the effects of vitamin D supplementation on inflammatory markers in patients with HF.

Research Design and Methods

This systematic review conforms to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines (Appendix 1) and is part of a wider evidence synthesis of the effects of vitamin D on inflammation in multiple diseases. The methods for this work were specified *a priori* in a published protocol[12], and a protocol for this meta-analysis was registered on PROSPERO (CRD:42016047753).

Data Sources and Search Strategy. Studies were identified by systematically searching electronic databases using relevant search terms (Appendix 2) and pre-specified criteria, as outlined in our protocol[12]. Literature was searched from inception to 25 January 2017 for human studies, with no date or language limits. The search was conducted using the following electronic databases: MEDLINE; Medline in-process and other non-indexed citations; CINAHL; EMBASE; and All Evidence Based Medicine (EBM) Reviews. Additional studies were sought manually by searching the National Institute of Health Clinical Trials (https://clinicaltrials.gov/) and Australian New Zealand Clinical Trials (https://www.anzctr.org.au) registries, and via reference lists from relevant studies.

Study screening and selection. Selection criteria using the PICOS (Population, Intervention, Comparison, Outcomes, Study design) framework established *a priori* were used to determine eligibility of articles as previously reported[12]. Eligibility criteria are outlined in Supplementary Table 1. Briefly, included studies were RCTs in any population with diagnosed HF, where the intervention was vitamin D supplementation provided in any form, dose, or route, compared to placebo or usual care, and with inflammatory marker outcomes. Studies conducted in participants without diagnosed HF or which did not use vitamin D supplementation were excluded. The selection process is outlined in Fig. 1. Two independent reviewers (AJR and AM) examined full-text articles to confirm eligibility, and consensus was resolved by discussion or referred to a third reviewer (BdC).

Data extraction. Two independent reviewers (AJR, AM) performed data extraction using a specifically developed template, which included study author, year, and design; sample size and demographics; randomization strategy; vitamin D regimen/s and co-intervention/s and type of control/comparator used; outcome definition and assessment; mean values of outcomes and their standard deviations or confidence intervals, point estimates, and measures of variability; frequency counts for dichotomous variables; and intention-to-treat analysis. Corresponding authors of included trials where required data were not presented were contacted to provide de-identified data (aggregated effect measures) for the purpose of meta-analysis.

Risk of bias and quality appraisal. Risk of bias was assessed at the study-level by two independent reviewers (AJR and AM) using a critical appraisal template (Appendix 3) with pre-specified criteria[12]. Individual quality items were examined using a descriptive component approach as previously described[12] with assessment of study design aspects (Appendix 3). Using this approach, each study was assigned a risk of bias rating (high, moderate, low, or insufficient information).

Quality of the evidence was assessed at the outcome-level using the grading of recommendations, assessment, development and evaluation (GRADE) approach[13]. Two independent reviewers (AJR and AM) graded quality of the evidence based on risk of bias, imprecision (upper or lower limit of 95% CI is > 0.5), inconsistency (heterogeneity), indirectness (heterogeneous participants, outcomes, or interventions), and suspicion of publication bias. Interpretation of the grading scores are presented in Supplementary Table 2. Disagreements were resolved by consensus.

Data synthesis and analysis. Data are presented in summary tables and in brief narrative form to describe the included studies. For meta-analysis, aggregate effect measures at the end of the intervention period were pooled into random-effects models and standardized mean differences (SMD) with 95% confidence intervals (95% CI) were computed since studies used different methods/assays and reported different inflammatory marker concentrations. Statistical heterogeneity was assessed using the I^2 test, where $I^2 > 50\%$ indicated moderate to high heterogeneity for which sensitivity analyses were applied. In sensitivity analyses, studies deemed as high or moderate risk of bias and/or which contributed to heterogeneity or had different participant characteristics (eg: studies in infants versus adults) were omitted to examine their effects on the results. For meta-analyses of more than two studies, visual inspection of funnel plots and Egger[14] and Begg[15] statistical tests were used to determine small study effects and potential publication bias. Meta-analyses were performed in Review Manager V.5.3.5 and publication bias was assessed using Comprehensive Meta-analysis Software V.3. P-values < 0.05 were considered statistically significant.

Data availability. The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Results

Outcomes of the search and screening process are presented in Fig. 1. Initial database searches for all RCTs of vitamin D supplementation (Appendix 2) yielded 9,762 records, of which 870 were duplicates. Abstracts and titles were screened for the remaining 8,892 records (including 194 grey literature records). Six records which were in HF patients with inflammatory marker outcomes were eligible for full-text assessment (Fig. 1). An additional four records were identified by manual searches and via clinical trials registries, totaling 10 records which were eligible for full-text review. Of these 10 studies, three were excluded with reasons outlined in Fig. 1, thus seven studies[16–22] met the inclusion criteria for qualitative synthesis.

Study characteristics. Descriptive data of the included studies are summarized in Table 1 and detailed in Supplementary Tables 3 and 4. All studies were in English-language and of parallel design. Study durations ranged from 6 weeks[19] to 12 months[17], with a mean duration of 7 months across the studies. Most studies enrolled older adults (age > 50 years), with one study investigating HF in infants[20] (Table 1).

Participant Characteristics. In studies of adult patients (n = 6 RCTs), the mean age of participants ranged from 62.7 to 80.6 years, while Schleithoff *et al.*[18] reported a median age of 57 and 54 years in vitamin D and placebo groups, respectively (Table 1). Males made up >50% of participants in the 6 studies reporting gender distribution. Mean/median baseline body mass index (BMI) as reported in five studies ranged from 25.4 to 34.8 kg/m². Mean and median baseline 25(OH)D concentrations ranged between 20.5–47.7 nmol/l and 35.9–48 nmol/l, respectively, as reported in six studies (Table 1). Only one study[21] explicitly excluded non-vitamin D-deficient participants [25(OH)D > 50 nmol/l]. Two studies reported HF duration (Table 1), while smoking status was reported in only three studies (Supplementary Table 4). Recruitment of participants was based on severity of symptoms determined by New York Heart Association (NYHA) classifications ≥II[16,18,21]; LVEF ≤ 35%, ≤40% or

Author, Year, Country	Design; Setting	N (n)*	Participants; (% male)	Intervention and Control arms	Frequency/ duration	Baseline age (y); BMI (kg/m²); and HF duration (months)		Baseline 25(OH)D (nmol/l)	Primary outcome/s	Biomarkers	Pooled
Boxer, 2014, USA	Parallel RCT; Academic HF and general cardiology practices	64 (64);	Adults > 50 y old with HF; (51% male)	I: 50,000 IU oral VD3 + 800 mg Ca; P: placebo + 800 mg Ca	Weekly 6 months	Age=	I: 65.8 ± 10.6 P:66.0 ± 10.4	I: 47.7 ± 7.5 P: 44.4 ± 22.5	RAAS	CRP	Yes
						BMI=	I: 34.8 ± 7.2 P: 31.3 ± 6.9				
						Duration=	NR				
McKeag, 2014, Northern Ireland	Parallel RCT; Hospital-based HF clinics	74 (74)	Adults with stable HF; (81% male)	I: 1,000 IU oral VD3 + 400 IU VD2; P: placebo (lactose)	Daily 12 months	Age=	I: 65.8 ± 9.4 P: 62.7 ± 9.0	I: 38.7 ± 13.8 P: 38.6 ± 23.7	LVEF, QoL, 6 min walk distance	IL-6, IL-10, TNF-α, CRP	Yes
						BMI=	I: 29.5 ± 2.4 P: 29.9 ± 5.9				
						Duration=	NR				
Schleithoff, 2006, Germany	Parallel RCT; Heart and Diabetes Centre	123 (93)	Adults with congestive HF; (83% male)	I: 2,000 IU oral VD3 + 500 mg Ca; P: Miglyol oil + 500 mg Ca	Daily 9 months	Age=	I: 57 (53, 63) P:54 (50, 62)	I: 35.9 (28.7, 55.2)P: 38.2 (31.7, 56.9)	Biochemical markers, LVEF, VO2 max	TNF-α, CRP, IL-6, IL-10	Yes
						BMI=	I: 26 (23.9, 29) P: 25.4 (24.3, 28.4)				
						Duration=	NR				
Schroten, 2013, Holland	Parallel RCT; Outpatient clinic	101 (94)	Adults chronic HF on optimal medical therapy; (93% male)	I: 2,000 IU oral VD3; P: NR	Daily 6 weeks	Age=	I: 63.5 ± 11.1 P: 64.0 ± 9.0	I: 46 (39, 63) P: 48 (38,61)	Plasma renin activity	Ngal, FGF-23	No†
						BMI=	NR				
						Duration=	I: 62 (34, 102) P: 61 (29, 133)				
Shedeed, 2012, Egypt	Parallel RCT; Teaching hospital cardiology unit of paediatric department	80 (80)	Infants with congestive HF; (61% male)	I: 1,000 IU oral VD3; P: placebo (dH2O)	Daily 3 months	Age=	I: 10.3 ± 4.6ª P: 11.2 ± 3.5ª	I: 33.5 ± 5.5 P: 34.9 ± 6.2	RAAS	IL-10, IL-6, TNF-α	Yes
						BMI=	N/A				
						Duration=	I: 5.39 ± 2.1 P: 5.11 ± 1.9				
Witham, 2010, UK	Parallel RCT; Primary and secondary care facilities	105 (84)	Older adults with chronic HF and low vitamin D (<50nmol/L); (66% male)	I: 100,000 IU oral VD2; P: NR	bolus doses quarterly (x3) 9 months	Age=	I: 78.8 ± 5.6 P: 80.6 ± 5.7	I: 20.5 ± 8.9 P: 23.7 ± 10.0	6 min walk, TUG, RAAS, BP	TNF-α	Yes
						BMI=	I: 27.2 ± 5.1 P: 27.3 ± 4.5				
						Duration=	NR				
Witte, 2005, UK	Parallel RCT; Community-based HF unit	28 (28)	Older >70 y adults with chronic HF due to ischemia; (NR% male)	I: 400 IU oral VD (type NR) + 250 mg Ca; P: NR	Daily 9 months	Age=	I: 74.2 ± 2.8 P: 75.5 ± 3.5	I: NR P: NR	LVEF, QoL, inflammatory cytokines	TNF-α, IL-6, TNFR-1, TNFR-2	Yes
						BMI=	I: 27.8 ± 2.4 P: 26.4 ± 3.5				
						Duration=	NR				

Table 1. Characteristics of studies included in systematic review of effects of vitamin D supplementation on inflammation in patients with heart failure. Data presented as mean ± standard deviation or median (interquartile range), unless otherwise specified. *N (n) = Number of participants randomized (number analyzed); †Unable to obtain all or some relevant outcome data from authors; ªdata represents months. Abbreviations: **HF**, heart failure; **RCT**, randomized controlled trial; **BMI**, body mass index; **VD3**, vitamin D3/ cholecalciferol; **VD2**, vitamin D2/ ergocalciferol; **Ca**, calcium; **IU**, international units; **I**, intervention group; **P**, placebo/control group; **BP**, blood pressure; **RAAS**, renin-angiotensin-aldosteron system; **LVEF**, left ventricular ejection fraction; **QoL**, quality of life; **VO2 max**, maximum volume of oxygen; **TUG**, Timed Up and Go test; **CRP**, C-reactive protein; **IL**, interleukin; **TNF-α**, tumor necrosis factor-alpha; **Ngal**, neutrophil gelatinase-associated lipocalin; **FGF-23**, fibroblast growth factor-23; **TNFR-1/-2**, tumor necrosis factor receptor-1/-2; **NR**, not reported; **N/A**, not applicable; **mo**, months; **y**, year.

<45%[19,20,22]; or both NYHA class II-III and LVEF ≤ 45%[17]. Only one study[16] reported ethnicity, where the proportion of African-Americans was approximately 63% of enrolled participants.

Intervention Characteristics. Oral cholecalciferol supplementation was used in five studies, with doses ranging from 1,000–2,000 IU daily[17–20] to a weekly dose of 50,000 IU[16]. Of these five studies, two co-supplemented oral cholecalciferol with calcium[16,18] and another supplemented 1,000 IU oral cholecalciferol in addition to 400 IU ergocalciferol daily as part of the Forceval© multivitamin supplement[17]. In the remaining two studies which did not use cholecalciferol[21,22], one used 100,000 IU of oral ergocalciferol administered three times over nine months[21] and the other did not specify the type of vitamin D used but stated an oral dose of 400 IU daily as part of a micronutrient[22].

Outcome Measures. Most studies reported LVEF[17,18,22], or renin-angiotensin-aldosterone-system (RAAS) activity[16,19–22] as primary outcomes. Various inflammatory markers were examined (Table 1), the most common of which was TNF-α, measured in 5 of the 7 RCTs. Other commonly measured markers included C-reactive protein (CRP) (n = 3 RCTs), interleukin (IL)-6 (n = 4 RCTs), and IL-10 (n = 3 RCTs) (Table 1).

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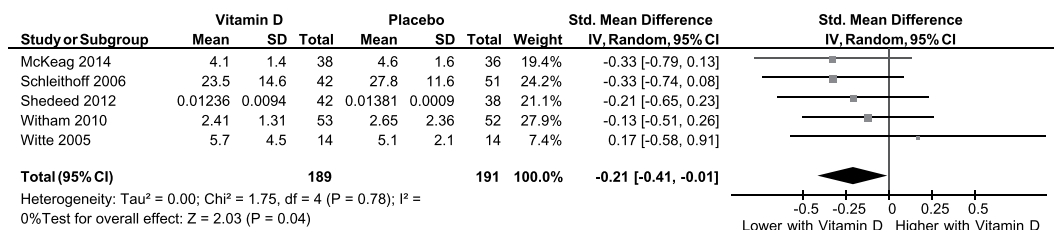
Figure 2. Forest plot showing results of a meta-analysis of the effects of vitamin D supplementation on tumor necrosis factor- *alpha*. Data are reported as SMDs with 95% CIs.

Risk of Bias assessment. Results of the risk of bias assessment are presented in Supplementary Table 5. All studies were double-blinded, except one which only employed single blinding of participants[19]. All studies reported dropout rates; however only three performed intention-to-treat analyses[16,17,20]. Selective reporting was evident in three studies[16,19,22]. Overall, most studies were rated as having high (n = 3) or moderate (n = 2) risk of bias, with three studies having low risk of bias.

Meta-analyses and sensitivity analyses. One of the seven studies[19] did not have available data for pooling and was excluded from meta-analysis. Data from the remaining six studies were pooled to examine differences in inflammatory markers between vitamin D and placebo groups at follow-up. Markers such as fibroblast growth factor-23 (FGF-23) and TNF-receptors had available data from single studies, and are therefore included in the descriptive analysis component.

Pooling of five RCTs (n = 380)[17,18,20–22] showed a significant difference in TNF-α concentrations between vitamin D and placebo groups at follow up [SMD (95%CI) = −0.21 (−0.41, −0.01); p = 0.04; I^2 = 0%; P_{het} = 0.8] (Fig. 2). In a sensitivity analysis, excluding the study in infants[20] attenuated the difference between vitamin D and placebo groups [SMD (95%CI): −0.21 (−0.44, −0.02); p = 0.07; I^2 = 0%; P_{het} = 0.6]. However, in a further sensitivity analysis excluding the study in infants[20] and including only low risk of bias studies[17,18,21], there was a significant difference in follow-up TNF-α concentrations between vitamin D and placebo groups [SMD (95%CI): −0.25 (−0.49, −0.01), p = 0.04; I^2 = 0%; P_{het} = 0.7].

Pooling of data from three studies (n = 231)[16–18] showed no significant difference between vitamin D and placebo groups in follow up CRP concentrations, with moderate heterogeneity [SMD (95%CI): −0.08 (−0.46, 0.30); p = 0.7; I^2 = 53%; P_{het} = 0.1] (Fig. 3A). Results remained non-significant in a sensitivity analysis limited to only low risk of bias studies[17,18] [SMD (95%CI): −0.20 (−0.69, 0.29); p = 0.4; I^2 = 61%; P_{het} = 0.1].

In pooled analysis of data from three studies (n = 247)[17,18,20], there were no significant differences in IL-10 concentrations between vitamin D and placebo groups at follow up [SMD (95%CI): 1.14 (-0.90, 3.19); p = 0.3; I^2 = 98%; P_{het} < 0.001], with significant heterogeneity (Fig. 3B). In a sensitivity analysis excluding the study in infants [20] which was also responsible for the significant heterogeneity, differences in IL-10 between groups remained non-significant[17,18] [SMD (95%CI): −0.16 (−0.52, 0.20); p = 0.4; I^2 = 28%; P_{het} = 0.4]. Both studies in this sensitivity analysis were deemed low risk of bias[17,18].

Similarly, pooled analysis of two studies (n = 154)[17,20] showed lower concentrations of IL-6 in the vitamin D group; however this was not statistically significant and heterogeneity was observed (SMD (95%CI): −2.00 (−5.65, 1.65); p = 0.3; I^2 = 99%; P_{het} < 0.001) (Fig. 3C). Given only two studies were included, further sensitivity analyses to explore or exclude sources of bias or heterogeneity were not possible.

Subgroup Analyses. Study and sample characteristics thought to be clinically relevant to the outcomes were assessed in subgroup analyses for TNF-α, CRP, and IL-10 as these markers had a sufficient number of studies (≥3 RCTs). Studies were stratified by dose (≤1000, or >1000 IU) and duration (≤6, or >6 months) of vitamin D supplementation and whether vitamin D was co-supplemented with calcium (>100 mg daily versus ≤100 mg or no calcium). There were no differences in TNF-α, CRP, or IL-10 between any of the subgroups (all p > 0.05; data not shown). Stratified analyses by baseline vitamin D status, BMI, or age were not possible since all studies had similar mean baseline 25(OH)D concentrations (<50 nmol/l) and mean ages and BMIs (>50 years and >25 kg/m2, respectively, except for the study in infants which was excluded in sensitivity analysis).

Descriptive Analyses. In a study that was excluded from meta-analysis due to unavailable data[19], 2000 IU daily of cholecalciferol for 6 weeks had no effect on FGF-23 or neutrophil gelatinase-associated lipocalin in patients with HF. Another study[22] measured IL-6 (data not available for meta-analysis) as well as TNF receptors 1 and 2 and found no effects after supplementation with 400 IU of oral vitamin D (type not specified) daily for 9 months.

GRADE assessment and Publication Bias. Based on visual inspection of funnel plots (Supplementary Figure 1), as well as Egger[14] and Begg[15] statistical tests (Supplementary Table 6), we found no evidence of publication bias for TNF-α, CRP, or IL10. Studies reporting on IL-6 could not be assessed for publication bias due to the small number of studies (n = 2 RCTs).

An evaluation of the quality of evidence using the GRADE approach[13] is presented in Supplementary Table 7. For TNF-α, the quality of evidence was high since most studies had low to moderate risk of bias with low

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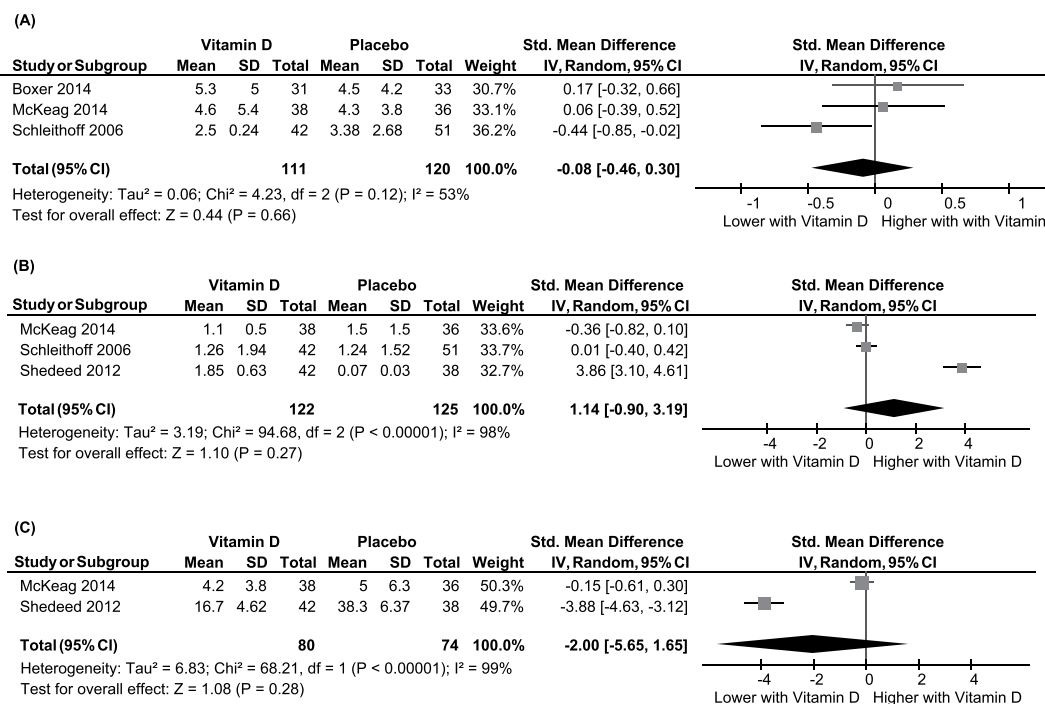
Figure 3. Forest plot showing results of a meta-analysis of the effects of vitamin D supplementation on C-reactive protein (**A**), interleukin-10 (**B**) and interleukin-6 (**C**). Data are reported as SMDs with 95% CIs.

statistical and clinical heterogeneity and narrow CIs. Moreover, although the effect for TNF-α was considered small (SMD < 0.5), it persisted in a sensitivity analysis including only low risk of bias studies. For CRP, quality of the evidence was deemed moderate due to imprecision (wide CI) and moderate heterogeneity. For IL6 and IL10, the evidence was deemed low quality due to high risk of bias, imprecision, and inconsistency ($I^2 > 90\%$ for heterogeneity), as well as having small numbers of studies and potential reporting bias (Supplementary Table 7).

Discussion
Summary of Findings.
In this systematic review and meta-analysis of RCTs in patients with HF, we found that vitamin D-supplemented groups had lower TNF-α concentrations, which persisted in a sensitivity analysis including only low risk of bias studies. There were no differences between vitamin D and placebo groups in CRP, IL-10 or IL-6. Our findings suggest that vitamin D may have specific, but modest effects on inflammation in patients with HF.

Summary of Previous Evidence.
Vitamin D is a pleiotropic steroid hormone, which elicits its functions by acting through the vitamin D receptor (VDR)[23]. The VDR is present in many cell types including cardiac myocytes[23]. In animal studies, mice defective in 1α-hydroxylase (the enzyme which converts inactive vitamin D to its active form) had altered calcium handling which led to exaggerated cardiac dysfunction consistent with HF in humans[24]. Additionally, these mice exhibited increased expression of the pro-inflammatory cytokines TNF-α and monocyte chemoattractant protein-1 (MCP-1), however a normal phenotype was restored upon supplementation with vitamin D, highlighting a potential role for vitamin D in HF pathogenesis[24].

Observational studies in humans support pre-clinical findings. Low serum 25(OH)D concentrations have consistently been observed in patients with HF, and have been associated with HF severity[25], and with inflammatory markers in HF[26]. Given these findings, RCTs sought to explore the clinical utility of vitamin D supplementation in improving inflammatory profiles in HF. As shown in our meta-analysis, vitamin D treatment may reduce circulating TNF-α concentrations; however, the observed effect was relatively small, and no study that contributed to this analysis[17,18,20–22] showed concomitant improvements in clinical or laboratory markers of HF such as LVEF or natriuretic peptides. Furthermore, a meta-analysis of seven RCTs in patients with HF[27] found that vitamin D supplementation did not improve clinical symptoms including LVEF, 6-minute walk test, and natriuretic peptide concentrations. It is therefore unclear if resolution of systemic inflammation can improve cardiac physiology and outcomes in patients with HF.

Our results support the findings of a previous meta-analysis by Jiang *et al.*[27], although our meta-analysis included a greater number of studies and markers, and a larger overall sample. Jiang *et al.*[27] reported no effects of vitamin D supplementation on IL-10 concentrations; however follow-up TNF-α and CRP concentrations were lower in vitamin D-supplemented groups compared with controls. Importantly, results for TNF-α in the meta-analysis by Jiang *et al.*[27] were based on pooled analysis of three RCTs (n = 257), while only two RCTs were pooled for CRP (n = 185). Here, we add to existing evidence by showing that the effect for TNF-α persisted in a larger meta-analysis of five RCTs totaling 380 patients, and importantly, that there was no effect on CRP when data from all three studies which measured CRP were pooled (n = 231). The present study also adds to current

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evidence by providing additional analysis of two RCTs (n = 154) reporting on IL-6, where we observed lower concentrations in the vitamin D group compared with placebo at follow-up, though this did not reach statistical significance. The observed effects, or lack thereof, persisted in sensitivity analyses of only low risk of bias studies. This adds robustness to our findings since sensitivity analyses were not performed in the previous meta-analysis[27].

Limitations of the Evidence.   Overall, the current literature is limited. Most studies had small samples, with <100 participants in all but one study (n = 101)[19]. Quality of the evidence across studies was low or moderate for most markers, and only the evidence for TNF-α was deemed high quality. Moreover, no study accounted for seasonal variation or sunlight exposure, and/or physical activity, which may potentially influence vitamin D levels, as well as body composition since vitamin D is fat-soluble and sequestered in adipose tissue[28]. Smoking status and HF duration were also not reported in several studies - factors which may influence inflammatory status in these patients. Finally, only one study actively recruited patients who were vitamin D-deficient at baseline[21]. Increasingly, it has been shown that beneficial effects of vitamin D supplementation are only observed when provided to vitamin D-deficient individuals[29]. Thus, future trials recruiting only vitamin D-deficient individuals may strengthen the evidence base, as could subgroup analyses comparing inflammatory marker profiles in vitamin D deficient versus replete individuals at follow-up. We were unable to perform subgroup analyses by baseline vitamin D status, or by studies that achieved adequate vitamin D status at follow-up due to the small number of studies and lack of reporting of follow-up vitamin D levels in most studies.

Study Strengths and Limitations.   Our study has some limitations. First, although most studies had low to moderate risk of bias, some high risk of bias studies included in the main analysis may have influenced the results. However, we performed sensitivity analyses where possible to account for the effects of high risk of bias studies. Moreover, randomization, blinding, and the use of a control group were considered the most important aspects in our meta-analysis, and most studies satisfied these criteria. Second, the studies identified were heterogeneous in terms of type of vitamin D used (cholecalciferol or ergocalciferol) and the dosing protocols, which may introduce some confounding to our analysis. We also could not ascertain whether biomarker values reported in each paper were derived from normal or skewed distributions, thus results should be interpreted with caution. Given the limited number of studies, we were unable to conduct subgroup analyses for certain study design aspects such as comparing daily versus monthly doses, and we were unable to perform meta-regression to account for other factors such as BMI or baseline vitamin D status of participants. Third, publication bias cannot be ruled out for markers with few studies or where we were unable to obtain all necessary data from authors. Finally, whilst our meta-analysis suggests that vitamin D may reduce TNF-α concentrations, the observed effect was small, and it remains unclear whether such effects would translate into improved health outcomes in patients with HF.

Nevertheless, we included only RCTs, the gold-standard for establishing causality. We applied rigorous international gold-standard methodology and conformed to international reporting standards with a protocol published *a priori* to ensure transparency. Our search strategy was comprehensive and included non-English language publications and grey literature. We sought data directly from authors in order to provide a more comprehensive meta-analysis with inclusion of further data and more inflammatory markers than the previous meta-analysis[27]. This meant that we were able to perform sensitivity analyses for some markers including TNF-α, not previously performed. These factors add robustness to our study and enable us to provide a comprehensive and up-to-date synthesis of current evidence of the effects of vitamin D supplementation on inflammatory markers in patients with HF.

Conclusions

In conclusion, we showed that vitamin D-supplemented groups had a small but significantly lower TNF-α concentration at follow-up compared with placebo. Although vitamin D may not be effective as a sole treatment to improve inflammation or HF outcomes, it may be beneficial as an adjunct to existing therapies in vitamin D-deficient patients with HF. However, further large-scale, well-designed trials including vitamin D-deficient participants and measuring both inflammatory markers and long-term clinical HF endpoints are needed to determine if vitamin D supplementation can reduce inflammatory markers and improve health outcomes for patients with HF.

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Acknowledgements

Author Contributions

A.J.R. conducted data extraction, quality appraisal, data synthesis and analysis, and drafted the manuscript. A.M. designed the protocol, performed the search, data extraction, quality appraisal, data synthesis and interpretation, and drafted the manuscript. D.S. and P.R.E. contributed to writing and editing the manuscript. Bd.C. determined the scope of the review, and contributed to protocol design and writing and editing the manuscript. Bd.C. had full access to the data, takes responsibility for data integrity, and is the guarantor of the review. All authors provided significant intellectual contributions to the manuscript and approved the final version for publication.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-19708-0.

Competing Interests: The authors declare that they have no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Title: Effects of vitamin D supplementation on inflammatory markers in heart failure: a systematic review and meta-analysis of randomized controlled trials
Authors: Alexander J. Rodriguez, Aya Mousa, Peter R. Ebeling, David Scott, Barbora de Courten

SUPPLEMENTAL MATERIAL

Supplementary Table 1. Eligibility criteria for study selection (PICOS)

	Participants (P)	**Intervention (I)**	**Comparison (C)**	**Outcomes (O)**
Inclusion	Males and female patients with heart failure diagnosed by NYHA class, LVEF, or brain natriuretic peptide, on any treatment regimen and for any duration, of any age, ethnicity, socio-economic status, geographic area, co-morbidity or pregnancy status	Any type of vitamin D supplementation (D2; D3; calcitriol; analoges) administered in any form (oral, intravenous, or intramuscular) alone or combined with other intervention/s, of any dosage, and for any duration	Placebo or usual care; any other non-pharmacological interventions or pharmacological interventions	Inflammatory biomarkers including but not limited to: all interleukins, all TNFα, TGF-β1, CRP, MCP-1, IFNγ, NFκB, MIF, fibrinogen, adipokines: leptin, resistin, visfatin, adiponectin, omentin
Exclusion	Studies in participants without diagnosed heart failure	Studies without vitamin D supplementation	Studies with no control/comparator group	Studies with no inflammatory marker outcomes measured
Study type (S)	Systematic reviews of RCTs and RCTs in humans			
Language	No limit			
Year of publication	No limit			

Abbreviations: NYHA, New York Heart Association; **LVEF**, left ventricular ejection fraction; **TNFα**, tumor necrosis factor-alpha; **TGF-β1**, transforming growth factor-beta 1, **CRP**, C-reactive protein; **MCP-1**, monocyte chemoattractant protein-1; **IFN-γ**, interferon-gamma; **NFκB**, nuclear factor kappa B; **MIF**, macrophage migration inhibitory factor; **RCTs**, randomized controlled trials.

Supplementary Table 2. Grading the quality of the evidence (adapted from GRADE Working Group, 2004)

Strength of Evidence	**Interpretation**
High quality	Very confident in the estimate of the effect and further research is very unlikely to change our confidence.
Moderate quality	Moderately confident in the estimate of the effect, but further research may have an important impact on our confidence and may change the estimate.
Low quality	Somewhat confident in the estimate of the effect, but further research is very likely to have an important impact on our confidence and will likely change the estimate.
Very low quality	Very little confidence in the estimate of the effect as it is very uncertain.

Supplementary Table 3. Study and Intervention Characteristics

Study details	n (analyzed)	Intervention and Control arms	Total VD Dose (IU)	Frequency/ Duration	Route	Participant Characteristics	Baseline 25(OH)D (nmol/l)	Primary outcome	Biomarkers
Boxer 2014, USA	64 (64)	**I:** 50,000 IU oral VD3 + 800 mg Ca; **P:** placebo + 800 mg Ca	50,000	Weekly 6 months	Oral Capsule	>50y males and females with HF	**I:** 47.7 ± 7.5 **P:** 44.4 ± 22.5	RAAS	CRP
McKeag 2014, Northern Ireland	74 (74)	**I:** 1,000 IU oral VD3 + 400 IU VD2; **P:** placebo (lactose)	1000 + 400	Daily 12 months	Oral Capsule	Adults with stable HF	**I:** 38.7 ± 13.8 **P:** 38.6 ± 23.7	LVEF, QoL, 6min walk distance	IL-6, IL-10, TNF-α, CRP
Schleithoff 2006, Germany	123 (93)	**I:** 2,000 IU oral VD3+ 500 mg Ca; **P:** Miglyol oil + 500 mg Ca	2000	Daily 9 months	Oral Capsule	Adults with congestive HF	**I:** 35.9 (28.7,55.2) **P:** 38.2 (31.7,56.9)	Biochemical markers, LVEF, VO2 max	TNF-α, CRP, IL-10
Schroten 2013, Holland	101 (94)	**I:** 2,000 IU oral VD3; **P:** NR	2000	Daily 6 weeks	Oral Capsule	Adults chronic HF on optimal medical therapy	**I:** 46 (39, 63) **P:** 48 (38,61)	Plasma renin activity	Ngal, FGF-23
Shedeed 2012, Egypt	80 (80)	**I:** 1,000 IU oral VD3; **P:** placebo (dH2O)	1000	Daily 3 months	Oral Oil drop	Infants with congestive heart failure	**I:** 33.5 ± 5.5 **P:** 34.9 ± 6.2	RAAS	IL-10, IL-6, TNF-α
Witham 2010, UK	105 (84)	**I:** 100,000 IU oral VD2; **P:** NR	100,000	3 doses (quarterly for 9 months)	Oral Capsule	Older adults with HF with low vitamin D (<50nmol/L)	**I:** 20.5 ± 8.9 **P:** 23.7 ± 10.0	6min walk, TUG, RAAS, BP	TNF-α
Witte 2005, UK	28 (28)	**I:** 400 IU oral VD (type NR) + 250 mg Ca; **P:** NR	400	Daily 9 months	Oral Capsule	Older >70y adults with HF due to ischemia	NR	LVEF, QoL, inflammatory cytokines	TNF-α, IL-6, TNFR-1, TNFR-2

Abbreviations: **HF**, heart failure; **RCT**, randomized controlled trial; **BMI**, body mass index; **VD3**, vitamin D3/cholecalciferol; **VD2**, vitamin D2/ ergocalciferol; **Ca**, calcium; **IU** international units; **I**, intervention group; **P**, placebo/control group; **BP**, blood pressure; **RAAS**, renin-angiotensin-aldosteron system; **LVEF**, left ventricular ejection fraction; **QoL**, quality of life; **VO2 max**, maximum volume of oxygen; **TUG**, Timed Up and Go test; **NT-proBNP**, N-terminal pro B-type natriuretic peptide; **CRP**, C-reactive protein; **IL**, interleukin; **TNF- α**, tumor necrosis factor-alpha; **8isoPGF2a**, 8-isoprotaglandin F2a ; **Ngal**, neutrophil gelatinase-associated lipocalin; **FGF-23**, fibroblast growth factor-23; **TNFR-1/-2**, tumor necrosis factor receptor-1/-2; **NR**, not reported; **N/A**, not applicable; **mo**, months; **y**, years.

Supplementary Table 4. Baseline participant characteristics and follow up biochemical analyses:

Study	n	Age (years)	Males n (%)	BMI (kg/m²)	HF duration (months)	Current Smokers n (%)	Follow Up 25(OH)D (nmol/l)	Follow Up CRP (mg/L)	Follow Up TNF-a (pg/ml)	Follow Up IL-6 (pg/ml)	Follow Up IL10 (pg/ml)	Follow Up FGF-23 (RU/mL)
Boxer	I: 31 P: 33	I: 65.8 ± 10.6 P: 66.0 ±10.4	I: 15 (48) P: 18 (54)	I: 34.8 ± 7.2 P: 31.3 ± 6.9	NR		NR	I: 5.3 ± 5.0 P:4.5 ± 4.2				
McKeag	I: 38 P: 36	I: 65.8 ± 9.4 P: 62.7 ± 9.0	I: 31 (82) P: 29 (81)	I: 29.5 ± 2.4 P: 29.9 ± 5.9	NR	I: 6 (16) P:10 (28)	I: 99.6 ± 23.8 P:35.4 ± 22.0	I:4.6 ± 5.4 P:4.3 ± 3.8	I: 4.1 ± 1.4 P: 4.6 ± 1.6	I:4.2 ± 3.8 P:5.0 ± 6.3	I: 1.1 ± 0.5 P:1.5 ± 1.5	
Schleithoff	I: 42 P: 51	I: 57 (53, 63) P:54 (50, 62)	I: 52 (85) P: 50 (80)	I: 26 (23.9,29) P: 25.4 (24.3, 28.4)	NR	I: 9 (14) P:7(11)	NR	I: 2.5 ± 0.24 P: 3.38 ± 2.68	I:23.5 ± 14.6 P:27.8 ± 11.6		I:1.26 ± 1.94 P:1.24 ± 1.52	
Schroten	I: 51 P: 50	I: 63.5 ± 11.1 P: 64.0 ± 9.0	I: 46 (90) P: 48 (96)	NR	I: 62 (34,102) P:61 (29,133)	NR	I: 80 (75, 87) P: 44 (39, 49)					I: 134 (114-159) P: 119 (105-136)
Shedeed	I: 42 P: 38	I: 10.3 ± 4.6[a] P:11.2 ± 3.5[a]	I: 27 (64) P: 22 (58)	I:8.6±1.6[b] P: 8.4±1.9[b]	I: 5.39 ± 2.1 P: 5.11 ± 1.9	N/A	I: 82.1 ± 5.7 P: 36.5 ± 16.0		I: 0.01236 ± 0.0094 P: 0.01381 ± 0.0009	I:16.7±4.62 P:38.3±6.37	I:1.85 ± 0.36 P: 0.07±0.03	
Witham	I: 42 P: 42	I: 78.8 ± 5.6 P: 80.6 ± 5.7	I: 34 (64) P: 35 (67)	I: 27.2 ± 5.1 P: 27.3 ± 4.5	NR	I: 8(15) P: 6(12)	NR		I: 2.41 ± 1.31 P: 2.65 ± 2.36			
Witte	I: 14 P: 14	I: 74.2 ± 2.8 P: 75.5 ± 3.5	NR	I: 27.8±2.4 P: 26.4±3.5	NR	NR	NR		I: 5.7 ±4.5 P: 5.1 ± 2.1			

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Data presented as mean ± standard deviation or median (interquartile range), unless otherwise specified.

Data not reported in published papers were obtained directly from corresponding authors.

[a]data represents months and [b]weight (g) instead of years or BMI, respectively, for study in infants.

Abbreviations: **HF**, heart failure; **BMI**, body mass index; **I**, intervention group; **P**, placebo/control group; **NR**, not reported; **N/A**, not applicable; **25(OH)D**, 25-hydroxyvitamin D; **CRP**, C-reactive protein; **IL**, interleukin; **TNF- α**, tumor necrosis factor-alpha; **FGF-23**, fibroblast growth factor-23.

Supplementary Table 5. Risk of Bias Assessment for Individual Studies

Study	Design*	Selection bias		Performance bias		Detection bias	Attrition bias		Reporting bias	Confounding		Other bias	Pooled in meta-analysis	ROB score
		Random sequence generation	Centralized /concealed allocation	Participants blinded	Investigators blinded	Outcome assessors blinded	Drop-outs reported	Intention to treat analysis	Free of selective reporting	Groups similar at baseline	Adequate statistical analysis	Funding/ COI reported		
Boxer, 2014	Parallel	NR	Yes	Yes	Yes	NR	Yes	Yes	No	Yes	No	Yes	Yes	Mod
McKeag, 2014	Parallel	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Partial	Yes	Yes	Low
Schleithoff, 2006	Parallel	Yes	NR	Yes	Yes	NR	Yes	No	Yes	Yes	Yes	Yes	Yes	Low
Schroten, 2013	Parallel	Yes	No	No	Yes	Yes	Yes	No	No	Yes	Yes	Yes	No[a]	High
Shedeed, 2012	Parallel	No	NR	Yes	Yes	NR	N/A	Yes	Yes	Yes	Partial	No	Yes	Mod
Witham, 2010	Parallel	Yes	Yes	Yes	Yes	NR	Yes	No	Yes	Yes	Yes	Yes	Yes	Low
Witte, 2005	Parallel	No	Yes	Yes	Yes	NR	Yes	No	No	No	No	NR	Yes	High

*all trials were parallel design RCTs (ie: randomized and with a control group) unless otherwise specified; [a]Unable to obtain all or some relevant outcome data from authors; Abbreviations: **COI**, conflict of interest; **ROB**, risk of bias; **NR**, not reported; **N/A**, not-applicable; **Mod**, moderate.

Supplementary Table 6. Egger and Begg statistical tests for assessment of publication bias

Inflammatory Marker	Number of Studies	Number of Participants	Egger's test*	Begg's test*	Beggs test* (continuity corrected)
TNF-α (ng/L)	5	380	0.26	0.62	0.80
CRP (mg/L)	3	231	0.18	0.11	0.29
IL-10 (pg/ml)	3	247	0.21	0.60	1.00
Il-6 (pg/ml)	2	154	NE	NE	NE

*Reports p-values calculated from Egger and Begg-Mazudumar tests for assessing small effect size.
Abbreviations: **CRP**, C-reactive protein; **TNF-α**, tumor necrosis factor-alpha; **IL**, interleukin; **NE**, not estimable.

Supplementary Table 7. GRADE assessment of the effect of vitamin D supplementation on inflammatory markers meta-analyses

Marker (Number of Studies)	Vitamin D n (%)	Placebo n (%)	Risk of bias	Inconsistency (heterogeneity)	Indirectness [a]	Imprecision [b]	SMD	Quality
TNF-α (3 RCTs)	189 (49.7)	191 (50.3)	No serious risk of bias	No serious inconsistency	Moderate indirectness	No serious imprecision	TNF-α levels were -0.21 (-0.41, -0.01) SDs lower in the vitamin D group compared to placebo	⊕⊕⊕⊕ High
IL-10 (3 RCTs)	122 (49.4)	125 (50.6)	Moderate risk of bias	Serious inconsistency	Moderate indirectness	Serious imprecision	No significant effect observed	⊕⊕ Low
CRP (3 RCTs)	111 (48.1)	120 (51.9)	No serious risk of bias	Moderate heterogeneity	No serious indirectness	Serious imprecision	No significant effect observed	⊕⊕⊕ Moderate
IL-6 (2 RCTs)	80 (51.9)	74 (48.1)	Serious risk of bias	Serious inconsistency	Serious indirectness	Serious imprecision	No significant effect observed	⊕⊕ Low

[a] Determined as serious where population, outcome measure, or intervention regimens (ie: co-supplementation or bolus versus single doses).vary significantly across studies.
[b] Determined as serious where the upper or lower 95% confidence interval is >0.5. Abbreviations: GRADE, grading of recommendations, assessment, development and evaluation; **CRP**, C-reactive protein; **TNF-α**, tumor necrosis factor-alpha; **IL**, interleukin; **SMD**, standardized mean difference; **SD/s**, standard deviation/s

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Supplementary Figure 1. Funnel plots for assessment of publication bias

Tumor necrosis factor-alpha:
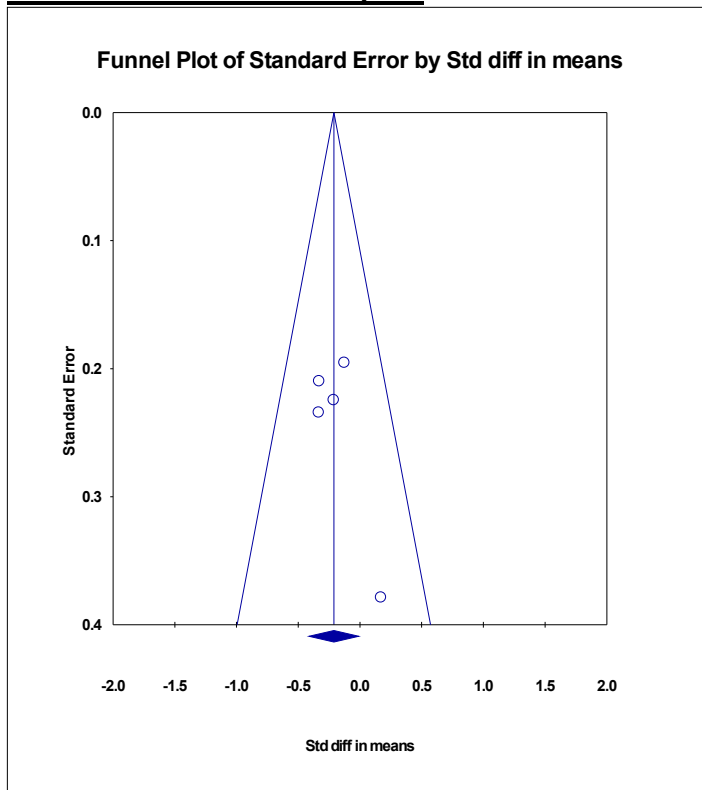
C-reactive protein:

Interleukin 10:

Funnel Plot of Standard Error by Std diff in means
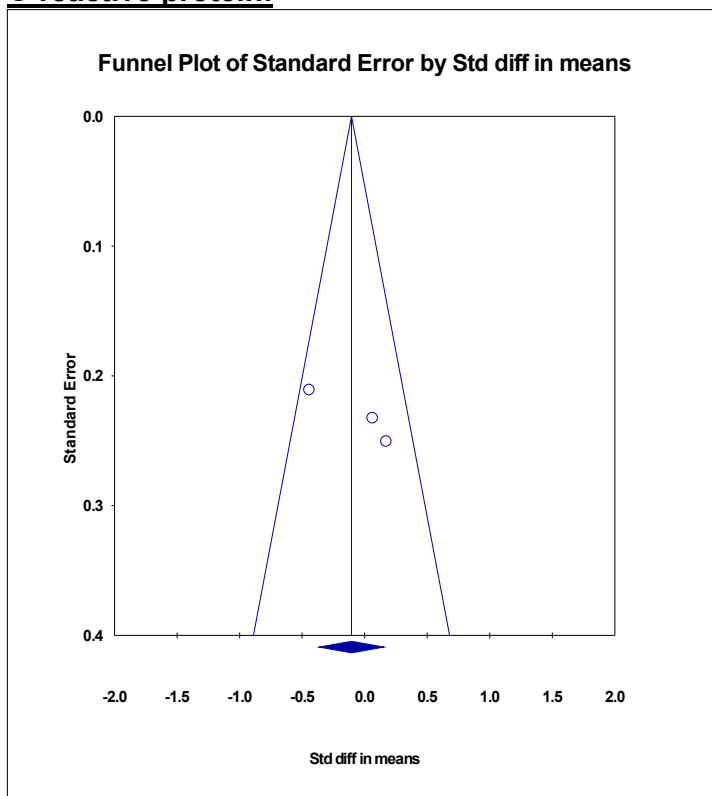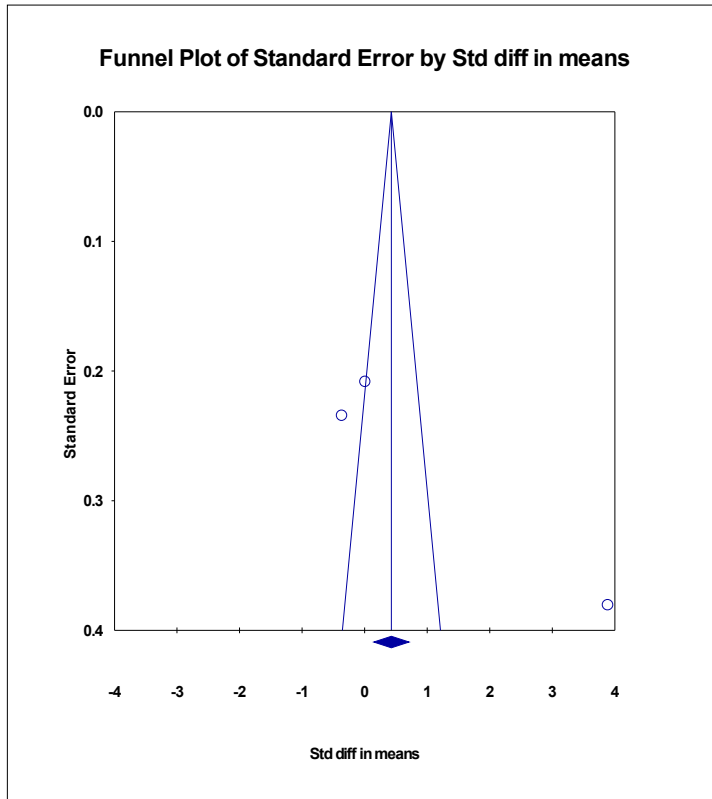
APPENDICES

Appendix 1. PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	3
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	4 [CRD:42016047753]
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	4 + Supplementary Table 1
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	4
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	4 (Appendix 2)
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	4
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	5
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	5 + Supplementary Table 1
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	5
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	5
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	6

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Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	5
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	6
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	6 + Figure 1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	6-7 + Table 1
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	7, 9-10
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	Figures 1-3, Table 1, Supplementary Tables
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	8-9
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	7, 9-10
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	9
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	10
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	12-13
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	11, 13
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	14

For more information, visit: **www.prisma-statement.org**.

Appendix 2. Sample OVID-MEDLINE search strategy

1. Vitamin D/	48. randomi?ed.ti,ab.
2. Vitamin D?.mp.	49. placebo.ti,ab.
3. 25OHD?.mp.	50. clinical trials as topic.sh.
4. 25 hydroxyvitamin D?.mp.	51. randomly.ti,ab.
5. 25-Hydroxyvitamin D3 1-alpha-Hydroxylase/	52. trial.ti.
6. 25-Hydroxyvitamin D? 1-alpha-Hydroxylase.mp.	53. or/46-52
7. 25 hydroxylase.mp.	54. exp animals/ not exp humans/
8. 24,25OHD?.mp.	55. 53 not 54
9. 24,25-Dihydroxyvitamin D3/	56. Meta-Analysis as Topic/
10. 24,25-Dihydroxyvitamin D?.mp.	57. meta analy$.tw.
11. exp Cholecalciferol/	58. metaanaly$.tw.
12. c?olecalciferol.mp.	59. Meta-Analysis/
13. Hydroxycholecalciferol/	60. (systematic adj (review$1 or overview$1)).tw.
14. hydroxyc?olecalciferol.mp.	61. exp Review Literature as Topic/
15. Dihydroxycholecalciferol/	62. or/56-61
16. Dihydroxyc?olecalciferol.mp.	63. cochrane.ab.
17. 1 alpha 25OHD.mp.	64. embase.ab.
18. 1a,25OHD?.mp.	65. (psychlit or psyclit).ab.
19. 1-alpha, 25 dihydroxyvitamin D?.mp.	66. (psychinfo or psycinfo).ab.
20. 1a, 25 dihydroxyvitamin D?.mp.	67. (cinahl or cinhal).ab.
21. 1-alpha hydroxylase.mp.	68. science citation index.ab.
22. 1-a, hydroxylase.mp.	69. bids.ab.
23. 1,25 hydroxyvitamin D?.mp.	70. cancerlit.ab.
24. 1,25 dihydroxyvitamin D?.mp.	71. or/63-70
25. 1,25 hydroxyc?olecalciferol.mp.	72. reference list$.ab.
26. 1,25 dihydroxyc?olecalciferol.mp.	73. bibliograph$.ab.
27. Calcitriol/	74. hand-search$.ab.
28. Calcitriol.mp.	75. relevant journals.ab.
29. Calcifediol/	76. manual search$.ab.
30. calcifediol.mp.	77. or/72-76
31. calciol.mp.	78. selection criteria.ab.
32. calcitetrol.mp.	79. data extraction.ab.
33. exp Ergocalciferol/	80. 78 or 79
34. ergocalciferol.mp.	81. Review/
35. ergosterol.mp.	82. 80 and 81
36. Dihydrotachysterol/	83. Comment/
37. dihydrotachysterol.mp.	84. Letter/
38. dihydrocalciol.mp.	85. Editorial/
39. alfacalcidol.mp.	86. animal/
40. paricalcitol.mp.	87. human/
41. vitamin D analogue.mp.	88. 86 not (86 and 87)
42. vitamin D analog.mp.	89. or/83-85,88
43. Ostelin.mp.	90. 62 or 71 or 77 or 82
44. Ostelin D?.mp.	91. 90 not 89
45. or/1-44	92. 53 or 91
46. randomi?ed controlled trial.pt.	93. 45 and 92
47. controlled clinical trial.pt.	94. limit 93 to humans

Appendix 3. Template for critical appraisal of randomised controlled trials:

Study ID	
Study citation	

EXTERNAL VALIDITY – IS THIS STUDY AND ITS RESULTS GENERALIZABLE TO MY SYSTEMATIC REVIEW QUESTION?		
Patient/population/ participants		
N	• Screened: • Enrolled: • Allocated/randomised: • Assessed: • Followed up: • Dropped out:	
Setting (hospital, clinic, community, university)		
Intervention/indicator (type, dose, duration, intervals)		
Comparison/control (type, dose, duration, intervals)		
Primary Outcome/s		
Secondary Outcome/s		
Inclusion Criteria	Yes No NR	
Exclusion Criteria	Yes No NR	
Does the study have a clearly focused question and/or PICO?	Yes Partial No NR	Consider if question is 'focused' in terms of: - population studied - intervention given/ exposure - comparison(s) - outcomes considered
Does the study have specified inclusion/exclusion criteria?	Yes Partial No	Consider if the inclusion or exclusion of patients was clearly defined a priori.
If there were specified inclusion/ exclusion criteria, were these appropriate?	Yes Partial No N/A	Consider if the eligibility criteria used to specify the patients, interventions/ exposures and outcomes of interest.
Were the outcomes measured appropriate?	Yes Partial No NR	Consider if the outcomes measured are appropriate and important outcomes.
Was there sufficient duration of follow-up?	Yes Partial No NR	May need to check with clinicians sufficient durations event occurrence.

INTERNAL VALIDITY – HAS THIS STUDY BEEN CONDUCTED RIGOROUSLY IN ORDER TO REDUCE BIAS?			
SELECTION BIAS	**Did the study have an adequate method of randomisation?**	Yes No NR	Method of randomisation is considered adequate when patient's allocation is entirely due to chance. Adequate methods include: - computer-generated random numbers - table of random numbers - coin tossing Inadequate methods include: - systematic methods (DOB, case record number, day of the week presenting) - sequence may be related to confounding variable - allows foreknowledge of assignment. (These studies should therefore be classed as Controlled Clinical Trials rather than RCTs.)
	Was allocation to intervention group concealed?	Yes No NR	Concealment of allocation is considered adequate when the person responsible for allocation cannot influence which group a patient is randomised to. Adequate methods of concealment of randomisation include: - Centralised or pharmacy-controlled randomisation - On-site computer based system with a randomisation sequence that is not readable until allocation - Other approaches with robust methods to prevent foreknowledge of the allocation sequence to clinicians and patients Inadequate approaches to concealment of randomisation - Open random numbers lists - Serially numbered envelopes (even sealed opaque envelopes can be subject to manipulation)
PERFORMANCE BIAS	**Were patients blind to intervention group?**	Yes No NR	Consider: - how the study has attempted to maintain blinding - if there is any indication that patients were aware of intervention group - the fact that blinding is not always possible - if every effort was made to achieve blinding
	Were investigators and care providers blind to intervention group?	Yes Partial No NR	Consider: - how the study has attempted to maintain blinding - if there is any indication that investigators or care providers were aware of intervention group - the fact that blinding is not always possible - if every effort was made to achieve blinding

	Aside from the experimental intervention, were the groups treated the same?	Yes Partial No NR	To be sure it's the intervention which is responsible for the effect.
DETECTION BIAS	**Were outcome assessors blind to intervention group?**	Yes Partial No NR	Consider: - If the outcome is objective (e.g. death) then blinding is less critical. - If the outcome is subjective (e.g. symptoms or function) then blinding of the outcome assessor is critical.
	Were all outcomes measured in a standard, valid and reliable way?	Yes Partial No NR	
	Were outcomes assessed objectively and independently?	Yes Partial No NR	Independence of assessment is important where the result of one outcome may affect the interpretation of another. When outcomes are objectively assessed, their independence from each other is less important.
ATTRITION BIAS	**What percentage of the individuals recruited into each arm of the study dropped out?**	$I=$ % $C_1=$ % $C_2=$ % $C_3=$ % NR	Consider: - if all patients who entered the trial were properly accounted for and attributed at its conclusion. - why patients dropped out, as well as how many. - the drop out rate may be expected to be higher in studies conducted over a long period of time.
	Were all the subjects analysed in the groups to which they were randomly allocated (ie intention to treat analysis)?	Yes No NR	Consider: - if analysis was as per protocol or intention to treat - number of crossovers - reason for crossover
REPORT BIAS	**Is the paper free of selective outcome reporting?**	Yes Partial No NR	Consider: - if all the planned outcomes were measured - if all the measured outcomes were reported - if any additional or composite outcomes were measured This is difficult to determine if there isn't a protocol.
CONFOUNDING	**Were the groups similar at baseline with regards to key prognostic variables?**	Yes Partial No NR	Key prognostic variable include age, sex, disease severity, inflammatory markers and vitamin D status. If the randomisation process worked, the groups should be similar, however particularly in small studies, some variations are very likely. There should be some indication of whether differences between groups are clinically important. May need to check with clinician.

	If confounding was present, was it controlled for?	Yes Partial No NR	Consider if any effort was made to control for confounding – Analyses were adjusted for:
	Were there any conflicts of interest in the writing or funding of this study?	Yes No NR	Consider: - if any of the authors are/were employed, sponsored etc by pharmaceutical companies, or have other financial/other ties - if any commercial companies were involved in funding, writing, editing, data analysis or manuscript approval
	Was the study sufficiently powered to detect any differences between the groups?	Yes Partial No NR	Consider: - if an adequate sample size calculation was undertaken - if the required sample size was recruited and retained - for which outcomes the study was powered - if confidence intervals include a clinically important difference, the study was underpowered NB: this is less important if significant differences were found
	For cross over studies - was the washout period adequate?	Yes No NR N/A	Consider the likely duration of action of the treatment being tested.
OTHER INTERNAL VALIDITY/BIAS	**If statistical analysis was undertaken, was this appropriate?**	Yes Partial No NR N/A	Consider: - whether the authors performed any statistical tests or just presented figures - if the statistical analysis was planned a priori- - if the data were analysed accordingly to the study protocol - the type of data and the statistical tests used. (Please refer to the CCE workbook as required) - use of parametric versus non-parametric tests; whether the data has been checked for normality - if the tests used are obscure, why did the authors used them and have they included a reference - if point estimates and measures of variability were presented for the primary outcome - if subgroups were analysed appropriately - if potential confounders were identified and taken into account in the analysis - if there was any adjustment made for multiple testing - if missing data was handled appropriately
Comments			*Add any other relevant comments, including if this is likely to influence the results of the study:*

| What is the overall risk of bias? | Low
Moderate
High
Insufficient information | *Low **-** All of the criteria have been fulfilled or where criteria have not been fulfilled it is very unlikely the conclusions of the study would be affected.*

Moderate - Some of the criteria have been fulfilled and those criteria that have not been fulfilled may affect the conclusions of the study.

High - Few or no criteria fulfilled or the conclusions of the study are likely or very likely to be affected.

Insufficient information – not enough information provided on methodological quality to be able to determine risk of bias. |

Cited in full as: Monash Centre for Health Research and Implementation (MCHRI) Evidence Synthesis Program template for critical appraisal of a randomised controlled trial (2013), MCHRI – Monash University and Monash Health, Melbourne, Australia (*adapted from* Critical Appraisal Templates (2010) Centre for Clinical Effectiveness, Southern Health, Melbourne, Australia).

Chapter 4.4: Original Research

Dietary calcium intake is associated with increased mortality and cardiovascular risk in men but not women

Rodríguez AJ, Scott D, Khan B, Khan N, Hodge H, English DR, Giles GG, Abrahamsen B & Peter R. Ebeling

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High calcium intake in men not women is associated with all-cause mortality risk: Melbourne Collaborative Cohort Study

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Abstract

Summary The risk of mortality associated with high dietary calcium is uncertain. Unlike a highly publicised study in Swedish women, high dietary calcium intake in men—not women—was associated with increased all-cause mortality.

Purpose The association of dietary calcium with mortality is controversial. A study of women from the Swedish Mammography Cohort (SMC) suggested higher calcium was associated with higher mortality risk, whilst a study of Australian adults from the Melbourne Collaborative Cohort Study (MCCS) suggested higher intakes were associated with lower mortality risk. Thus, we aimed to perform a sex-specific re-analysis of the MCCS to evaluate the association of dietary calcium with mortality outcomes and directly compare hazard estimates (95% confidence intervals) in women with those from the SMC.

Methods A prospective cohort study of community-dwelling Australian adults was conducted, in which 34,627 individuals (women 20,834 (60.2%); mean ± SD, age = 54 ± 8 years) were included at baseline after excluding those with prevalent cardiovascular (CV) disease, cancer or incomplete data. Energy-adjusted dietary calcium was categorised into the following levels of consumption (mg/day): < 600, 600–999, 1000–1399 and ≥ 1400. Mortality from all-causes, any cardiovascular disease and myocardial infarction was determined. Mortality hazards relative to intakes were estimated to be of 600–999 mg/day.

Results In women, hazard estimates for calcium intake of ≥ 1400 mg/day did not reach significance for all-cause (HR = 0.85; 0.66, 1.10) or CV (HR = 1.10; 0.69, 1.81) mortality in adjusted models. In men, intakes of ≥ 1400 mg/day were associated with a 42% increased all-cause mortality risk (HR = 1.42; 1.02, 1.99). There was a trend toward increased CV mortality (HR = 1.83; 0.94, 3.55).

Conclusion Contrary to findings from a similar study conducted in Swedish women, Australian women, after adjustment for cofounders showed no increase in mortality risk with high calcium intakes possibly reflecting differences in calcium handling dynamics, diet or lifestyle factors between the two countries. We identified an increased risk for men.

Keywords Calcium · All-cause mortality · Cardiovascular disease · Diet · Cohort study

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Introduction

Calcium plays a central role in bone health and also has several extra-skeletal functions, most notably in the cardiovascular system. Controversy surrounds whether increased calcium intake leads to an increase in the risk of incident cardiovascular events and mortality. Some randomised studies have reported an increased risk of cardiovascular events from calcium supplementation, and subsequent meta-analysis has seemingly confirmed that supplementation appears to be responsible for increased event rates [1, 2]. Naturally, this has driven clinical attention toward ensuring adequate calcium intake from dietary sources. However, it remains unclear if dietary calcium can deliver reductions in cardiovascular events and mortality. Recent data from two, large and rigorously designed prospective cohort studies in diverse population groups have generated conflicting results [3, 4]. As part of the Swedish Mammography Cohort (SMC), over 60,000 postmenopausal Swedish women with a mean age of approximately 53 years at enrolment were followed up over a median of 19 years [3]. In a fully adjusted proportional hazard model, a high calcium intake (> 1400 mg/day) from dietary sources was associated with higher all-cause mortality (hazard ratio (HR) = 1.40; 95% confidence interval 1.17, 1.67) and higher cardiovascular mortality (HR = 1.49; 1.09, 2.02). These models were fitted with a reference group of 600–999 mg/day in line with Swedish guidelines on recommended daily calcium intakes. Conversely, in the Melbourne Collaborative Cohort Study (MCCS) including over 40,000 women and men in an urban population (~25% were non-Anglo-Celtic) with a mean age of 54 years at enrolment, higher intakes of dietary calcium (median 1076 mg/day) were associated with a reduced risk of all-cause mortality (HR = 0.86; 0.76, 0.98) and cardiovascular disease mortality (HR = 0.84; 0.70, 0.99) relative to the lowest intake group with a median intake of 641 mg/day [4].

A number of key differences between these two studies may explain the contrasting findings. First, the SMC study estimated hazard relative to calcium intakes between 600 and 999 mg/day to model risks relative to the recommended intake of 800 mg/day in Sweden. The MCCS, however, estimated hazards relative to the lowest quartile of calcium intake. Second, the median energy-adjusted calcium intake was higher in the top group in the Swedish study (1260 mg/day) than in the Australian study (1076 mg/day) as the Australian study excluded individuals with calcium intakes more than three standard deviations above the cohort mean. Therefore, there may be some effect at the extremes of calcium intake not captured as part of the MCCS investigation. In this regard, it is interesting to note the upper quartile of calcium intake in the MCCS corresponded to the second highest intake group in the SMC [3]. Finally, the SMC consisted entirely of women whilst in the MCCS analysis, men and women were analysed together. Given the oversampling of women in the MCCS

(approximately 60%), sex-specific effects of dietary calcium on mortality may influence the combined result for women and men together.

Thus, the association of dietary calcium intake with the risk of mortality and cardiovascular disease remains unclear and is the source of continued scientific debate despite a number of well-conducted meta-analyses on the topic [5–8]. Thus, our objectives were fourfold: (1) to examine sex-specific associations of dietary calcium intake on mortality outcomes given that the MCCS oversampled women and the SMC suggested effects in women, (2) to directly compare risk estimates in women between the MCCS and SMC and explore potential explanations for any differences, (3) to examine the effects of dietary calcium intake on mortality outcomes including individuals with calcium intakes more than 3 standard deviations above the mean as there may be some effect at the extremes of calcium intake not captured in the original MCCS study and (4) to estimate hazards relative to calcium intakes of 600–999 mg/day to harmonise the statistical approach to the SMC.

Methods

Study design and population

The MCCS has been described in detail previously [9, 10]. Briefly, the MCCS is a prospective cohort study investigating dietary patterns and lifestyle factors on their impact on the incidence of cancer and mortality that enrolled 41,514 participants (17,045 men; 24,469 women) aged 40–69 at baseline (1990–1994). The sample was largely of Anglo-Saxon origin, but approximately 25% were born in Southern Europe. Participants were recruited by personal letters of invitation, using electoral registers, advertisements and community announcements in local media. The Cancer Council Victoria and Melbourne Health Human Research Ethics Committees approved the study (2008.212), and all participants gave written, informed consent.

For the present analysis, we excluded participants who did not complete a baseline food frequency questionnaire (FFQ) (n = 46, < 0.01%) or had reported a history of angina, myocardial infarction, stroke, diabetes mellitus or cancer at baseline (n = 6839, 16.4%). However, we did not exclude participants whose dietary calcium intake was more than three standard deviations above the sex-specific means (as per our previous analysis in this cohort) in order to determine whether effects on mortality are observed in the extremes of calcium intake as suggested in the SMC [3].

Following exclusions, a total of 34,627 (20,834 women; 13,793 men) participants were eligible for mortality and cardiovascular event analysis. As previously described [9], structured face-to-face interviews were used to obtain information on lifestyle and sociodemographic factors, including country

of birth, smoking, physical activity, alcohol intake, educational attainment, history of hypertension and arthritis, age and sex. Height and weight were measured, and body mass index (kg/m^2) was calculated.

Calcium intake

Diet during the 12 months preceding baseline was assessed using a 121-item FFQ [11]. Total energy and nutrient intakes were calculated from Australian food composition tables [12]. Calcium supplementation was defined as use at least once weekly. Total calcium intake could not be calculated because data on supplement dose and frequency were not obtained. An Australian Government survey at the time of baseline (1994–1995) revealed that only 7% of women (no data for men) reported taking calcium supplements and that up to 66% of calcium was obtained from dietary sources in the survey population. This suggests dietary sources were the primary contributor to calcium intake [13]. Dietary calcium intake was adjusted for energy intake using Willett's residual method as previously described [14]. Using these adjusted values, men and women in the cohort were divided into groups of calcium intake (mg/day) defined by the intake thresholds from the Swedish study by Michaëlsson et al. which were G1 < 600, G2 = 600–999, G3 = 1000–1399 and G4 ≥ 1400 [3].

Death ascertainment

Deaths to 11 August 2016 were identified from the Victorian Registry of Births, Deaths and Marriages and the National Death Index. Underlying cause of death was coded by the Australian Bureau of Statistics. For these analyses, we used deaths from all causes, any CV disease (CVD) (ICD-9 390–459 or ICD-10 I00–I99) and myocardial infarction (MI) (ICD-9 410–412 or ICD-10 I21–I23). Our previous analysis had mortality data up until 31 December 2006 [4].

Statistical approach

Clinical and demographic information was presented for men and women (Table 1) and calcium intake groups (Supplementary Tables 1 and 2). Cox proportional hazard regression models estimated HRs and 95% confidence intervals (95% CIs) in relation to calcium intake for all-cause mortality and mortality from cardiovascular disease and myocardial infarction across calcium intake groups. Follow-up began at baseline and ended (censored) at death, date left Australia or 11 August 2016, whichever came first. Model covariates were chosen as previously described by Khan et al. [4] which did not violate the proportional hazard assumptions and included the following: age (years), BMI (kg/m^2), region of birth (Australia/UK, Italy/Greece/other), area code-based relative socioeconomic status (Socio-Economic Indices for Areas

(SEIFA), categorised into quintiles from the most disadvantaged to the least disadvantaged) [15], physical activity (categorised using a score combing frequency and intensity of exercise in the past 6 months), educational level attained (never attended school/some primary school/completed primary school/some high or technical school/completed high or technical school/other qualification/some study toward tertiary degree/tertiary degree or diploma), usual daily alcohol consumption (g/day), hypertension (yes/no), smoking (never/former/current), fat intake (g/day), sodium intake (mg/day), phosphorous intake (mg/day), vegetable intake (categorised into groups according to the number of serves (0–3; 4–5; 6–7; 7+) per day, fruit intake (categorised into groups according to the number of serves (0–2; 2–3; 4–5; 6+) per day and use of calcium supplements (yes/no). Hazard ratio estimates were calculated relative to the second group of calcium intake in order to replicate the approach in the SMC. The rationale provided by Michaëlsson et al. for this was that the second quartile encompassed the recommended daily intake of 800 mg/day in Sweden [16]. Values for HR were considered significant if the confidence intervals did not cross 1.00. These point estimates were plotted in a forest plot to visualise the difference in risk between calcium intake groups compared to those of the SMC. We initially ran the model for men and women together and tested for an interaction of which there was not ($p = 0.58$, log-likelihood ratio test); however, we justified a separate analysis based on the known biological differences between men and women and known differences in life expectancy in addition to our desire to directly compare mortality in Australian and Swedish women. Additionally, we fitted spline curves to model the relationship between the multivariable-adjusted HR and energy-adjusted calcium intake as a continuous variable. A restricted cubic spline was constructed with knots placed at percentiles 5, 35, 65 and 95 in order to estimate the change in risk profiles across the various calcium intake levels, again referenced at 800 mg/day. A number of sensitivity analyses were conducted, stratifying the cohort by the use of calcium supplements across groups of social disadvantage and excluding those with extremely high calcium intake (more than 3 standard deviations above the sex-specific mean) to investigate potential sources of bias. All data were analysed using Stata v14.2 (StataCorp, College Station, Texas, USA).

Results

Clinical and demographic participant characteristics at baseline

Included in this analysis are 20,834 (60.2%) women and 13,793 (39.8%) men following exclusions (Table 1). Detailed between-group calcium intake differences in demographic, nutritional and clinical characteristics for women and

Table 1 Clinical, nutritional and demographic information of the cohort

	All (n = 34,627)	Women (n = 20,834)	Men (n = 13,793)
Adjusted calcium intake (mg/day)	865.30 [416.94]	972.84 [391.24]	702.86 [401.72]
Ethnicity (n[%])			
Anglo-Celtic	26,279 [75.89]	16,127 [77.5]	10,152 [72.6]
Southern European	8348 [24.11]	4707 [22.6]	3641 [26.4]
Age (years)	54.61 [8.54]	54.53 [8.44]	54.72 [8.69]
Height (cm)	165.01 [9.36]	159.93 [6.70]	172.69 [7.37]
Weight (kg)	73.03 [13.5]	67.94 [12.16]	80.71 [11.74]
BMI (kg/m^2)	26.79 [4.37]	26.62 [4.83]	27.07 [3.57]
WC (cm)	84.91 [12.75]	79.53 [11.55]	93.03 [9.85]
SBP (mmHg)	136.11 [18.82]	134.26 [19.27]	138.91 [17.76]
DBP (mmHg)	76.08 [11.59]	72.76 [10.93]	81.09 [10.73]
HTN (n [%])	3309 [9.56]	1159 [5.6]	2150 [15.6]
Ever smoked: yes/no (n [%])	14,242 [41.13]	6341 [30.4]	7901 [67.2]
Smoker (n [%])			
Never	20,384 [58.87]	14,493 [69.56]	5892 [42.71]
Former	3902 [11.27]	1854 [8.90]	2048 [14.85]
Current	10,340 [29.86]	4487 [21.54]	5853 [42.44]
Alcohol intake (g/day)			
0	14,830 [43.1]	1098 [53.0]	3850 [28.2]
1–39 (male)/1–19 (female)	16,114 [46.9]	7897 [38.1]	8217 [60.2]
40–59 (male)/20–39 (female)	2479 [7.2]	1522 [7.3]	957 [7.0]
60+ (male)/40+ (female)	955 [2.8]	327 [1.6]	628 [4.6]
Total energy intake (kJ/day)	9307 [3748]	8555 [3371]	10,443 [3994]
Protein (g/day)	99.94 [43.00]	100.03 [41.83]	99.89 [43.75]
Fat (g/day)	83.17 [37.82]	76.80 [33.82]	92.81 [41.35]
Carbohydrates (g/day)	251.07 [114.54]	235.57 [107.55]	274.48 [120.65]
Fibre (g/day)	31.05 [13.41]	30.17 [12.81]	32.36 [14.16]
Sodium (mg/day)	3160 [1363]	2932 [1207]	3505 [1505]
Potassium (mg/day)	3951 [2027]	3953 [2008]	3950 [2040]
Calcium (mg/day)	864.84 [417.80]	857.71 [414.16]	875.60 [423.02]
Phosphorus (mg/day)	1755 [857]	1673 [816]	1880 [901]
Calcium supplement use (n [%])	3765 [10.87]	3397 [16.3]	368 [2.7]
Dairy product intake (number of serves/week)			
0	8393 [24.2]	4608 [22.1]	3785 [27.4]
1	8549 [24.7]	5018 [24.1]	3531 [25.6]
2	8906 [25.7]	5567 [26.7]	3339 [24.2]
3+	8779 [25.3]	5641 [27.1]	3138 [22.7]
Type of milk used (n [%])			
Full cream	12,168 [35.1]	6163 [29.5]	6005 [43.5]
Reduced fat	13,449 [38.8]	8562 [41.1]	4887 [35.4]
Skim	5715 [16.5]	4247 [20.3]	1468 [10.6]
Soy	900 [2.6]	592 [2.8]	308 [2.2]
I do not use milk	2395 [6.9]	1270 [6.1]	1125 [8.1]
Vegetable intake (number of serves/day)			
0–3	4570 [13.2]	1951 [9.4]	2619 [19.0]
4–5	10.453 [30.19]	5699 [27.4]	4754 [34.5]
6–7	9983 [28.83]	6423 [30.8]	3560 [25.8]
7+	9621 [27.78]	6761 [32.5]	2860 [20.7]

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Table 1 (continued)

	All (*n* = 34,627)	Women (*n* = 20,834)	Men (*n* = 13,793)
Fruit intake (number of serves/day)			
0–2	5140 [14.84]	2395 [11.5]	2745 [19.9]
2–3	12,345 [35.65]	7026 [33.7]	5319 [38.6]
4–6	9060 [26.16]	5844 [28.1]	3216 [23.3]
6+	8082 [23.34]	5569 [26.7]	2513 [18.2]
Fish intake (number of serves/day)			
0–1	6710 [19.38]	3956 [19.0	2754 [20.0]
1–1.5	8102 [23.40]	4861 [23.3]	3241 [23.5]
1.5–2.5	11,630 [33.59]	7030 [33.7]	4600 [33.4]
2.5+	8185 [23.64]	4987 [23.9]	3198 [23.2]
Physical activity score (*n* [%])			
0 (least)	7732 [22.33]	3179 [23.0]	4553 [21.9]
1–3	7021 [20.28]	2564 [18.6]	4457 [21.4]
4–5	12,012 [34.69]	4512 [32.7]	7500 [36.0]
6+ (most)	7862 [22.70]	3538 [25.7]	4324 [20.8]
Socioeconomic disadvantage (*n* [%])			
1 (most)	6109 [17.75]	3745 [18.1]	2364 [17.3]
2	7157 [20.79]	4325 [20.9]	2832 [20.7]
3	5439 [15.80]	3200 [15.4]	2239 [16.3]
4	6382 [18.54]	3812 [18.4]	2570 [18.8]
5 (least)	9336 [27.12]	5637 [27.2]	3699 [27.0]
Educational level attained (*n* [%])			
Never attended school	201 [0.58]	167 [0.80]	34 [0.25]
Some primary school	2192 [6.33]	1490 [7.15]	702 [5.09]
Completed primary school	4175 [12.06]	2438 [11.70]	1737 [12.59]
Some high/technical school	13,094 [37.81]	8917 [42.80]	4177 [30.28]
Completed high/technical school	3437 [9.93]	1921 [9.22]	1516 [10.99]
Other qualification	2480 [7.16]	1153 [5.53]	1327 [9.62]
Some study tertiary degree/diploma	1173 [3.39]	604 [2.90]	569 [4.13]
Completed tertiary degree/diploma	7875 [22.74]	4144 [19.89]	3731 [27.05]

men are given in Supplementary Tables 1 and 2, respectively. Of note, there were a higher prevalence of hypertension (15.6% vs. 5.6%) and smoking (67.2% vs. 30.4%) amongst men and a higher prevalence of daily calcium supplementation taken daily amongst women. Also, men had higher daily intakes for alcohol, protein, fat, carbohydrates, sodium and phosphorous. The proportion of individuals consuming different numbers of serves of vegetables and fruit was similar between men and women, though women tended to consume more fruit and vegetables. Levels of physical activity undertaken and socioeconomic disadvantage were similar between men and women. Concerning calcium sources between men and women, a higher proportion of women consumed three or more dairy products a week and men tended to consume full cream milk whereas women consumed more reduced fat milk and skim milk. A higher proportion of men consumed no dairy products during the week.

Deaths at follow-up

The average follow-up time was approximately 12.5 years. In women, there were 2788 deaths from all-causes, 649 deaths from cardiovascular disease and 140 deaths related to myocardial infarction. In men, there were 2965 deaths from all causes, 741 deaths related to cardiovascular disease and 181 deaths from myocardial infarction. Person-years at risk, crude rate of death per person-years at risk and crude hazard ratios are given in Supplementary Tables 3 and 4.

Mortality risks in women

Relative to the second group (600–99 mg/day) as per Michaëlsson et al., the highest intake quartile (≥ 1400 mg/day) was associated with an approximately 49% increased cardiovascular mortality risk in a crude model but the

Table 2   Adjusted hazard ratios (HRs) in women and men according to the Swedish threshold for calcium intake

	Energy-adjusted calcium intake (mg/day)							
	< 600		600–999		1000–1399		≥ 1400	
	n (women/men) = 2547/5421		*n* (women/men) = 9602/5941		*n* (women/men) = 6410/1893		*n* (women/men) = 2275/538	
	Events	HR [95% CI]	Events	HR [95% CI]	Events	HR [95% CI]	Events	HR [95% CI]
Women								
All-cause mortality	396	0.96 [0.79, 1.18]	1297	Ref	790	0.94 [0.81, 1.09]	300	0.85 [0.66, 1.10]
Cardiovascular mortality	102	1.20 [0.80, 1.78]	295	Ref	175	1.06 [0.79, 1.41]	76	1.11 [0.69, 1.81]
Myocardial infarction	25	1.65 [0.65, 4.18]	62	Ref	35	0.90 [0.43, 1.91]	18	0.79 [0.22, 2.76]
Men								
All-cause mortality	1281	1.01 [0.88, 1.15]	1203	Ref	365	1.09 [0.90, 1.33]	115	*1.42 [1.02, 1.99]*
Cardiovascular mortality	319	0.86 [0.64, 1.16]	295	Ref	92	1.25 [0.84, 1.86]	34	1.83 [0.94, 3.55]
Myocardial infarction	76	0.95 [0.49, 1.87]	77	Ref	20	1.54 [0.66, 3.59]	8	1.84 [0.34, 9.91]

Italics denote 95% confidence interval does not cross unity

significance did not persist in a fully adjusted model (Table 2). No other risk estimate for any outcome was associated with any significant benefit or harm. When modelled as a continuous variable in a spline curve of hazard estimates, higher calcium intakes were not associated with significantly elevated mortality risks for any outcome and confidence intervals were wide. Particularly concerning cardiovascular and myocardial infarction mortality, the slope of the curve tended to increase with increasing calcium (Supplementary Figures 1–3).

Mortality risks in men

Relative to the second quartile (600–999 mg/day), the highest intake quartile (≥ 1400 mg/day) was associated with an approximately 42% increased all-cause mortality risk in a multivariable-adjusted model (95% CI 1.02, 1.99). No other significant hazard estimate was observed for other outcomes (Table 2). When modelled as a continuous variable in a spline curve of hazard estimates, higher calcium intakes were not associated with significantly elevated mortality risks for any outcome and confidence intervals were wide. Concerning all-cause mortality, the slope of the curve tended to increase with increasing calcium but this trend was not apparent regarding cardiovascular and myocardial infarction mortality (Supplementary Figures 4–6).

Sensitivity analyses

In a sensitivity analysis whereby those with a calcium intake greater than 3 standard deviations above the sex-specific mean were excluded as per the previous MCCS analysis, a number of the results changed. In women, 256 individuals were excluded from the analysis. No hazard estimate reached

statistical significance for any outcome (Supplementary Table 5). In men, there were 86 individuals excluded from analysis. This resulted in a number of hazard estimates for multiple outcomes reaching statistical significance. With these outliers excluded, the highest quartile (≥ 1400 mg/day) of calcium intake was associated with an approximately 54% increased all-cause mortality (95% CI 1.08, 2.19) and an approximately twofold increased cardiovascular mortality risk (HR = 2.25; 95% CI 1.16, 4.37) in fully adjusted models though the confidence interval was wide regarding cardiovascular mortality (Supplementary Table 6). Further sensitivity analyses were conducted to explore the potential influence of socioeconomic status and the use of calcium supplements on mortality outcomes (Supplementary Tables 7 and 8). There was no apparent trend in mortality risks concerning social disadvantage. However, in women with the least social disadvantage, calcium intakes above 1400 mg/day were associated with an approximately 48% reduced all-cause mortality risk (HR = 0.52; 0.30, 0.90; 81 events in 723 individuals). In contrast, in men with the least social disadvantage, calcium intakes above 1400 mg/day were associated with an approximately 86% increased all-cause mortality risk (95% CI 1.08, 3.21; 36 events in 167 individuals) and calcium intakes between 1000 and 1399 mg/day were similarly associated with an approximately 86% increased all-cause mortality risk (95% CI 1.16, 2.97; 72 events in 377 individuals). Similarly, the use of calcium supplements did not appear to influence mortality risks in women; however, in men, calcium intakes above 1400 mg/day in those that did not consume calcium supplements were associated with an approximately 44% increased all-cause mortality risk (95% CI 1.02, 2.02; 112 events in 511 individuals; Supplementary Table 9).

Comparison with previously published estimates

In line with the aims of this study, we directly compared both the crude and multivariable-adjusted hazard estimates in women relative to the second calcium intake group. These estimates were presented as forest plots (Figs. 1 and 2) to facilitate comparison. In crude models, it appears that in this cohort, there was no apparent trend regarding all-cause mortality but, in previously published data from SMC, there was a clear U-shaped relationship [3] (Fig. 1). This observation persisted in multivariable-adjusted hazard models (Fig. 2). Regarding CV and MI mortality, in both crude (Fig. 1) and multivariable-adjusted (Fig. 2) models, both the MCCS cohort and the SMC cohort exhibited U-shaped relationships in mortality risks.

Discussion

This re-analysis of our previously published data from the MCCS demonstrated that, in women, there was no significant mortality risk associated with increasing calcium intake after adjustment for multiple confounders. In men, calcium intakes above 1400 mg/day were associated with an approximately 42% increased risk of all-cause mortality relative to intakes of 600–999 mg/day. Moreover, in our sensitivity analyses excluding individuals with extremely high calcium intakes in men, the hazard estimate for all-cause mortality increased in magnitude and the estimate for cardiovascular mortality reached statistical significance albeit with a wide confidence margin. In women, no hazard estimate reached significance. Spline curves, which modelled calcium intake as a continuous variable, indicated that the very highest calcium intakes had wide confidence intervals and were not associated with increased risk in men or women. Importantly, these extremes of calcium intake values may not be realistic (the maximum energy-adjusted calcium intake was 6368 mg/day) and may be a result of probable errors in completing the FFQ by participants which may lead to extremely high values. Additionally, men were not as likely to be in the highest calcium intake group of > 1400 mg/day [2275 (10.9%) women versus 538 men (3.9%)], meaning the risk estimates for this intake group in men were based on fewer events than in other intake groups.

Our findings contradict the results from the SMC study, on which we based our analysis. In the SMC which included only women, calcium intakes above 1400 mg/day were associated with an approximately 40% increased all-cause mortality risk. However, women from the present study had a non-significant 17% (95% CI = 0.64, 1.08) reduced risk of all-cause mortality. Given that we applied the same calcium intake thresholds, calculated the hazard estimates relative to intakes of between 600 and 999 mg/day and did not exclude individuals with
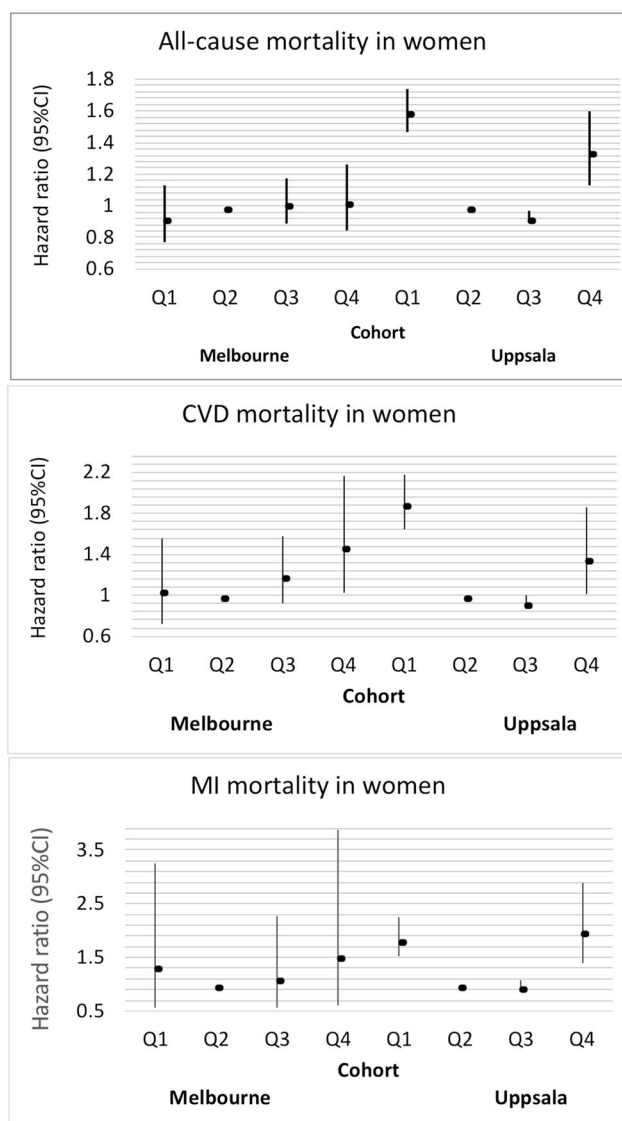
Fig. 1 Comparison of crude point estimates for mortality between MCCS and SMC cohorts

calcium intakes above 3 standard deviations from the mean as per the SMC strategy, it is unclear what could account for these contrasting risks between these women. However, a number of differences in baseline characteristics between the MCCS and SMC are apparent. It appears that BMI, prevalence of current smokers, fat intake and total energy intake were consistently higher in the MCCS across all intake groups compared to women of the SMC. However, there were more calcium supplement users in the MCCS in all groups except the group consuming greater than 1400 mg/day. This suggests that those with the highest intakes in the SMC were obtaining their calcium predominantly from non-dietary sources, and this was explored by Michaëlsson et al. Given that these differences largely paint the MCCS as being more "unhealthy" in a classic cardiovascular risk reduction framework, it is further surprising we were unable to replicate the SMC findings.
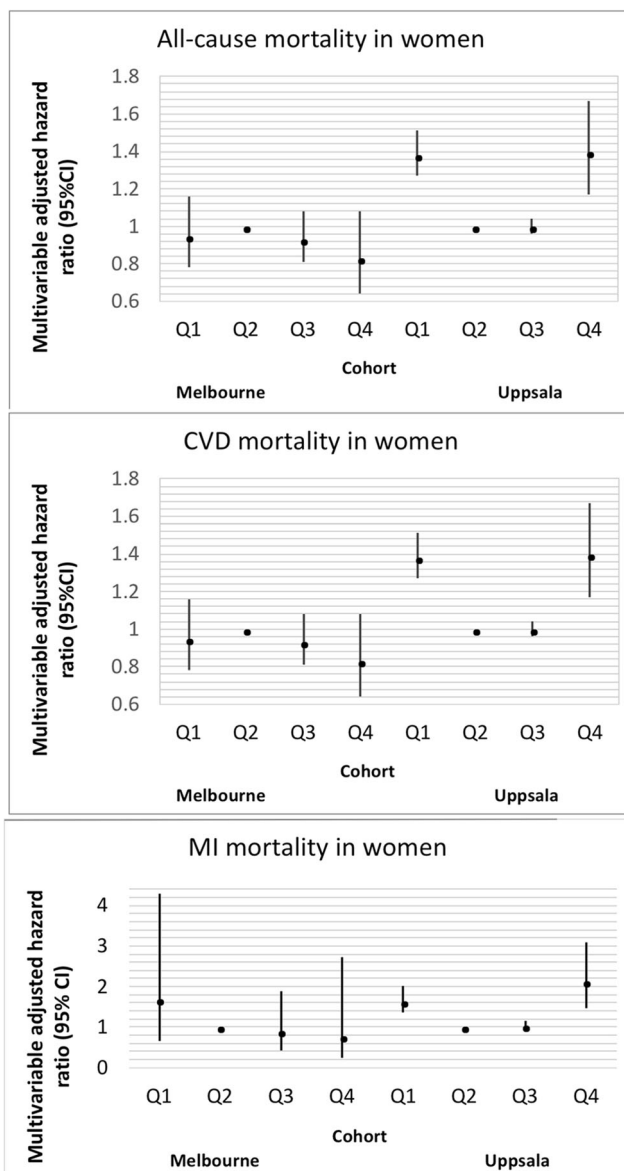
Fig. 2 Comparison of adjusted point estimates for mortality between MCCS and SMC cohorts

Of importance is the potential for sunlight exposure to influence blood vitamin D levels and, subsequently, the uptake of calcium. Despite the prominence of vitamin D deficiency/insufficiency in Melbourne, the vitamin D due to UV exposure will be greater in Melbourne and this could contribute to differences between the study findings, but we cannot account for this [17].

The MCCS, whilst largely of Anglo-Celtic origin, had approximately 25% of participants who were born in Southern Europe. No information was provided by the SMC investigators as to the ethnic make-up of their cohort though it is assumed to be predominantly Caucasian. Previous epidemiological research has suggested that there are differences in dietary calcium consumption between different ethnic groups

particularly in men which could contribute to mortality risks, but this is yet to be demonstrated in large cohorts [18]. Lactose malabsorption appears to be more prevalent in non-Anglo-Celtic populations which may contribute to low dietary calcium consumption [19, 20]. As such, calcium may be obtained through supplemental sources but, in our sensitivity analyses, the results of investigating those that did and did not use supplemental calcium did not alter our conclusions in women. Importantly, the overall prevalence of calcium supplement use was low in this cohort as consumption of calcium supplements was not popular during the baseline years of the study. Also, the median intake in the highest intake group (above 1400 mg/day) in the SMC was approximately one fifth greater than the highest intake group of the MCCS from our previous investigation. It is possible that the calcium intakes in the MCCS are not high enough to contribute to mortality risk. What could be driving the apparent sex-specific risk? Preclinical studies have shown that there are sex-specific differences in calcium handling dynamics of heart muscle in response to calcium which impacted on contractility and this may underlie observations of sex-specific mortality risks from our study [21, 22]. Furthermore, women were more likely to respond to invitation and participate in the study which leads them to being over-represented (approximately 60% of the MCCS). Whereas, previously, we saw that higher calcium intake was associated with reduced all-cause mortality risk, when analysed separately, men appear to have increased risk and women appear to have (non-significant) reduced risks associated with higher calcium intake. As women appear to be protected by high calcium intake in the present sex-specific analysis, this may account for the apparent contrasting sex-specific results compared with our previous findings for the entire cohort [4].

What are the underlying mechanisms by which calcium intake may influence mortality? Low calcium intake may be indicative of poorer health behaviours [23], and thus, associations between low calcium intake and increased mortality outcomes may not, in fact, be causal but instead reflect residual confounding by factors related to poor lifestyle and exposures to other risk factors. High calcium intake, on the other hand, has been shown to transiently increase serum calcium and supress parathyroid hormone, which may increase the calcification susceptibility of the vascular endothelium [24, 25]. Vascular calcification, related to endothelial dysfunction, is robustly associated with cardiovascular and all-cause mortality [26]. Other studies, however, have shown no such association, and thus, further research is warranted with respect to this [27, 28]. Additionally, excess circulating calcium may interfere with normal vitamin D homeostasis, increasing inflammatory cytokines and upregulating the renin-aldosterone-angiotensin system which is involved in the pathogenesis of a range of conditions such as cardiac hypertrophy, hypertriglyceridaemia and endothelial

dysfunction, all of which may increase mortality through various mechanisms [29].

Our study was limited by its cohort design, which may be subject to residual confounding. Any differences in risk estimates between our analysis and the SMC data, despite employing largely similar statistical methods, could be attributable to our analysis not adjusting for variables not captured in our analysis but were in the SMC, including the Charlson comorbidity index, nulliparity and living alone. Importantly, the large contributor to the heterogeneity between our analysis and the previous is the use of different instruments used to measure diet. The FFQ employed in this study has not been validated to determine sodium intake which may have an influence on cardiovascular outcomes. Also, our study included assessment of dietary calcium intake at only one time point whereas the SMC investigation applied time-updated data and regression calibration to improve accuracy of the exposure. Additionally, we did not quantify supplemental calcium intake and different sources of calcium may potentially impact mortality in different ways [30]. Supplemental calcium has increased in popularity, and calcium consumption patterns may have changed over time. Although information was collected on whether a multivitamin was taken as this practice is common in the Australian population, we were unable to determine if these multivitamins contained additional calcium or vitamin D or other substances that may potentially influence calcium or mineral metabolism. Prevalence of lactase enzyme deficiency is unknown in this population, and this information was not captured in the questionnaires. Whilst the present cohort was large and diverse, it was not designed to be fully representative of the wider Australian population especially considering recent migration patterns to Australia particularly from countries for which low calcium intake is common. Participants in the study were likely more health conscious and may have higher dietary calcium intake than the general population. Further, some outcomes had low event rates across the intakes groups which limited the investigation of the risk associated with high or low calcium intake and the risk estimates for these outcomes have wide confidence intervals. Our study has a number of strengths including its large sample size, many years of follow-up, robust ascertainment of deaths and possibility of adjusting for many covariates that could potentially impact on calcium biology, nutrition, cardiovascular disease and mortality.

In conclusion, a higher calcium intake was associated with a higher risk of mortality in men, but not in women after adjustment for multiple confounders. Spline curves suggested that the extremes of calcium intake may not be driving this risk. Identifying this safe range will help guide clinical and public health decision making. Much uncertainty remains as to the exact effect of dietary calcium on mortality, but our data suggests these effects may be sex-specific and regional/ethnic-specific. Our results are in contrast to findings in Swedish women suggesting higher calcium consumption was associated with increased mortality risks. Rather than seeking to determine discrete cut-off values to determine higher or lower risk, it appears sensible to identify a range of safe calcium intakes to recommend for both men and women.

Compliance with ethical standards

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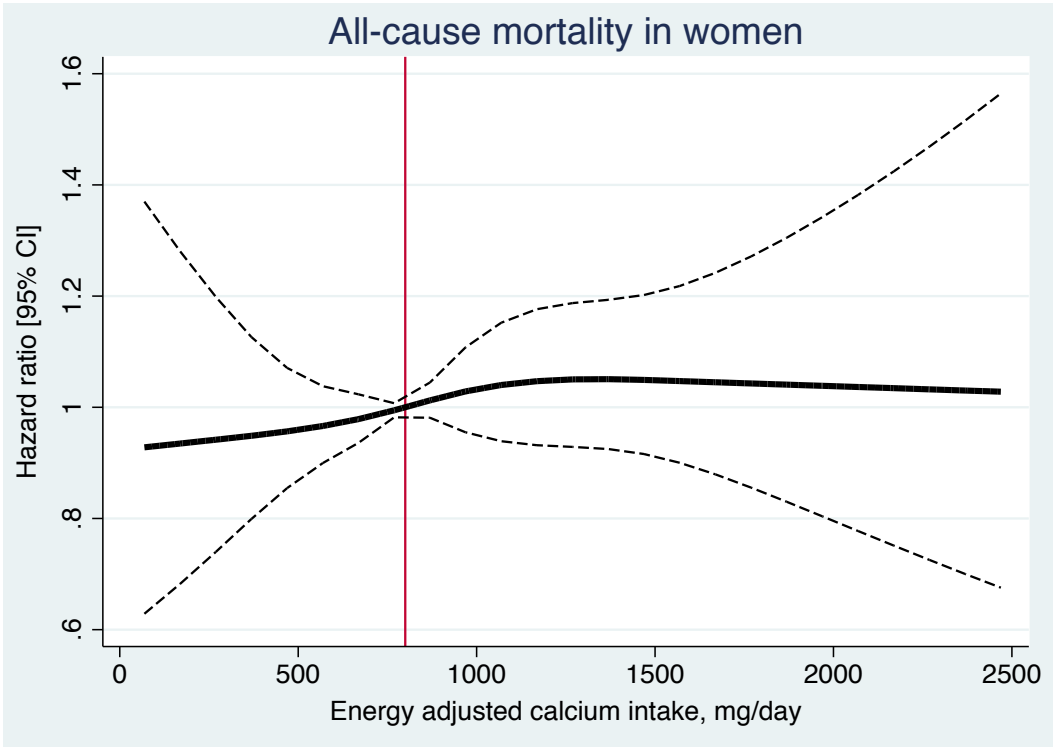
Supplementary figure 1. Spline curve modelling hazard for all-cause mortality in women
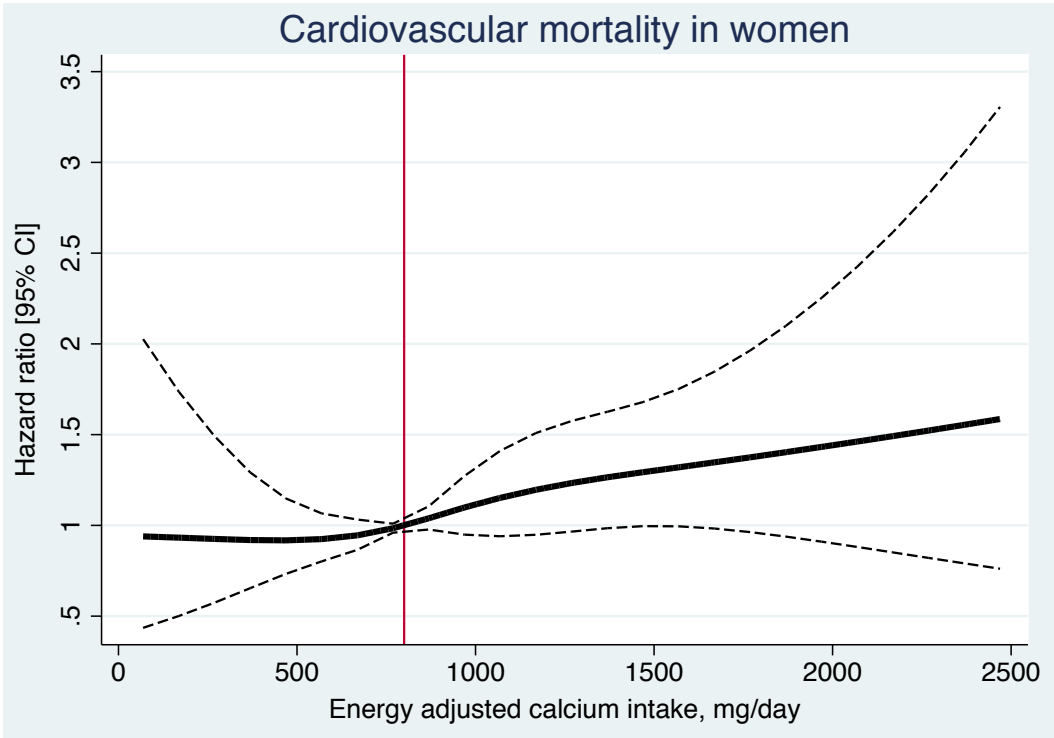
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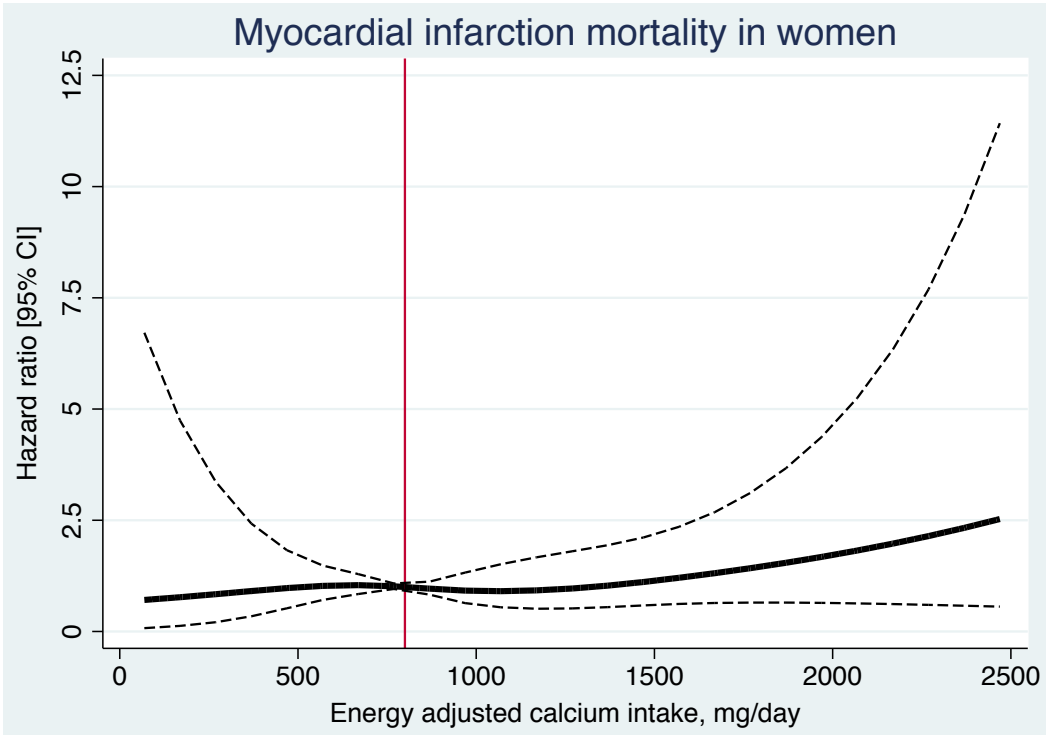
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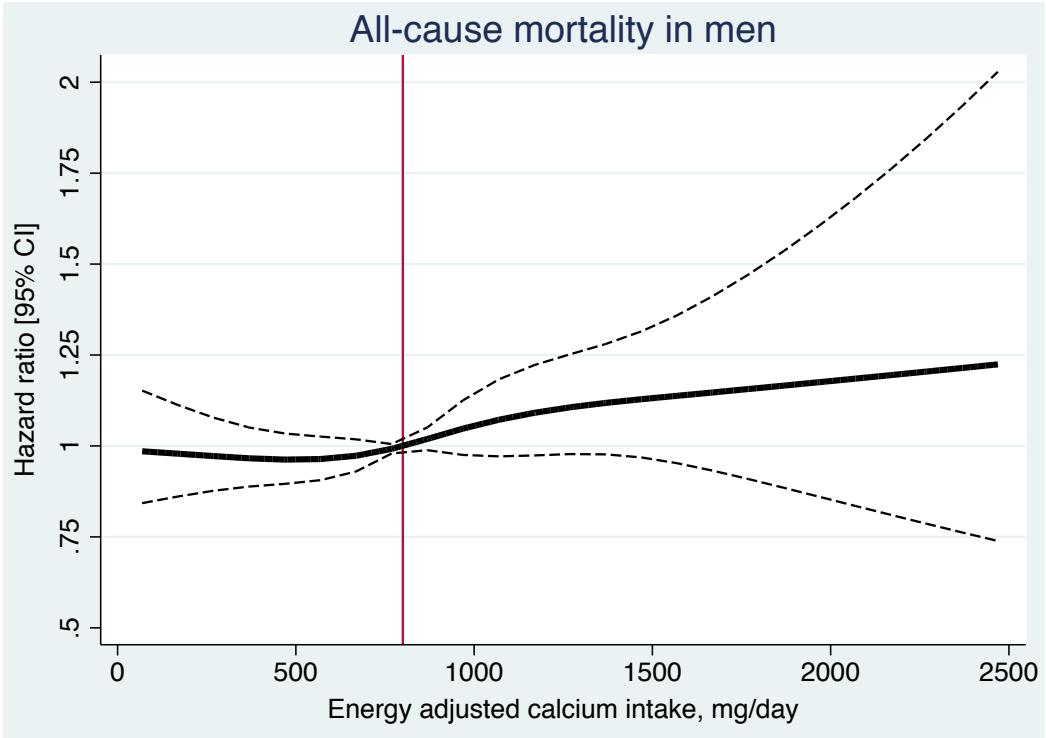
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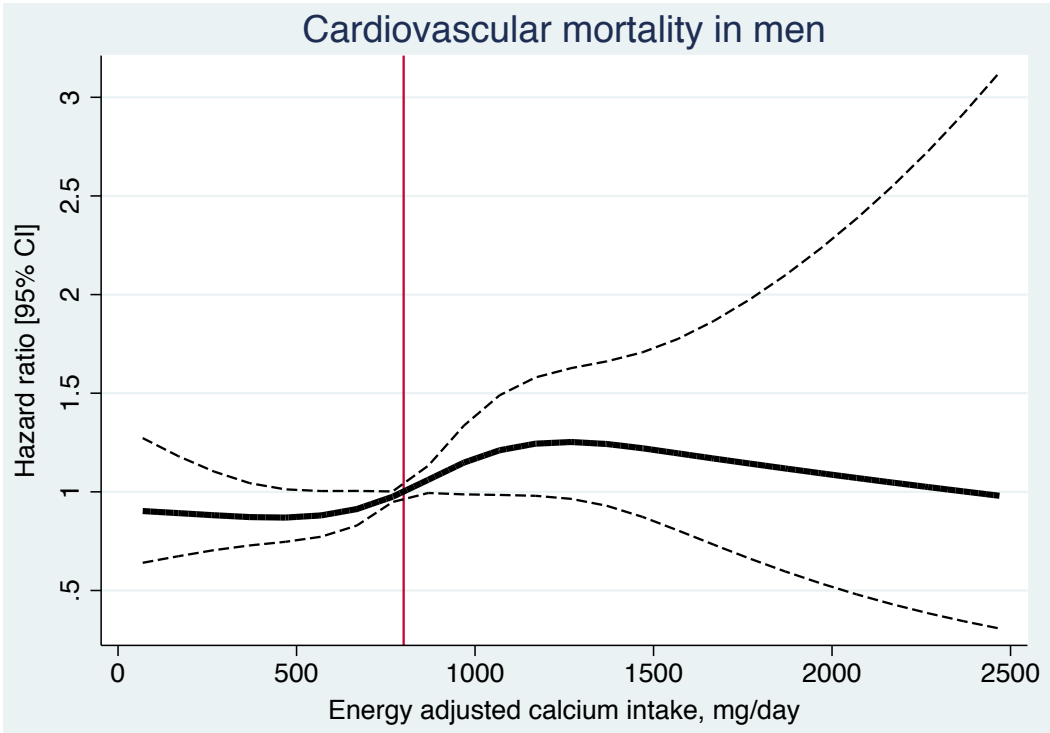
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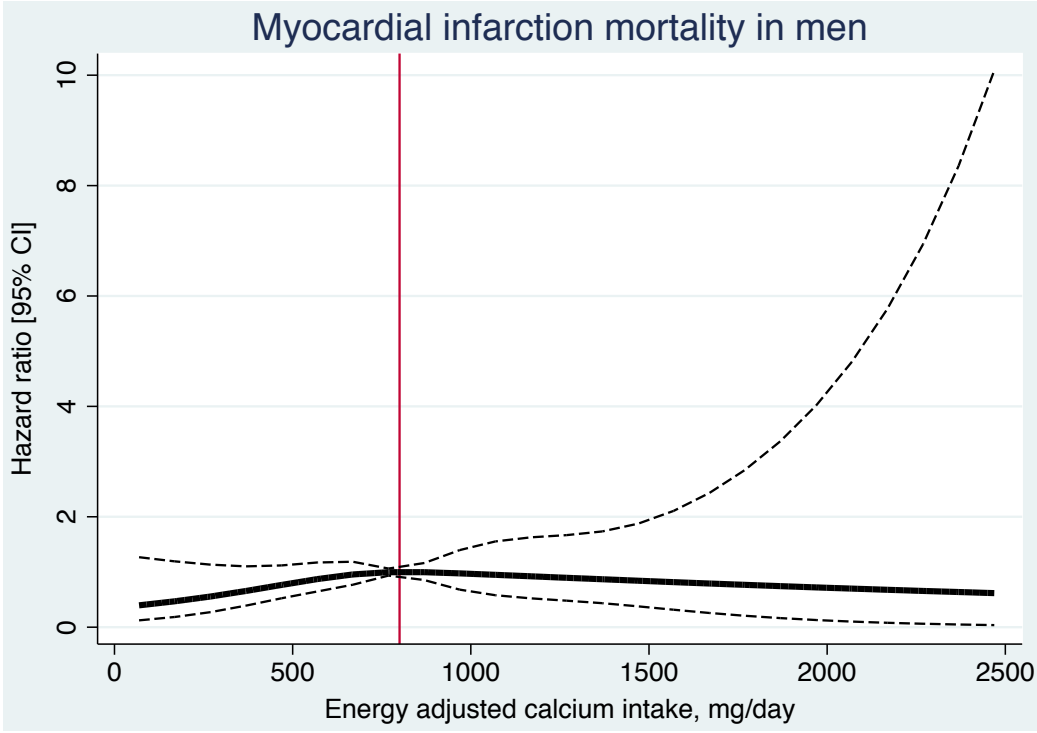
Supplementary figure 6. Spline curve modelling hazard for MI mortality in m

Supplementary Table 1. Clinical, nutritional and demographic information of men in the cohort

Calcium intake group (mg/day)	<600	600-999	1000-1399	≥1400
n	5421	5941	1893	538
Adjusted calcium intake (mg/day)	354.11[240.70]	780.92[111.94]	1153.79[105.26]	1772.82[469.01]
Ethnicity (n[%])				
Anglo-Celtic	3614[66.7]	4520[76.1]	1579[82.9]	448[83.3]
Southern European	1807[33.3]	1421[23.9]	323[17.1]	90[16.7]
Age (years)	55.0[8.5]	54.7[8.7]	54.6[8.8]	54.3[9.0]
Height (cm)	172.0[7.35]	172.84[7.44]	173.48[7.13]	173.99[6.90]
Weight (kg)	81.2[11.9]	80.6[11.6]	79.4[11.3]	80.1[11.2]
BMI (kg/m^2)	27.4[3.6]	26.9[3.5]	26.4[3.43]	26.4[3.3]
WC (cm)	94.38[9.83]	92.71[9.84]	90.89[9.64]	91.48[9.39]
SBP (mmHg)	140.57[17.92]	138.34[17.73]	136.97[17.32]	137.61[17.58]
DBP (mmHg)	82.00[10.71]	80.78[10.72]	79.91[10.53]	79.72[11.04]
HTN (n[%])	1082[19.9]	966[16.2]	284[15.0]	79[14.6]
Ever smoked – yes/no (n[%])	344[63.5]	3262[54.9]	924[48.8]	271[50.4]
Smoker (n[%])				
Never	1977[36.4]	2678[45.0]	969[51.2]	267[49.6]
Former	1056[19.4]	753[12.6]	177[9.35]	62[11.5]
Current	2388[44.0]	2509[42.2]	747[39.4]	209[38.8]
Alcohol intake (g/day)				
0	1192[22.27]	1767[29.98]	694[37.01]	197[37.10]
1-39 (male) / 1-19 (female)	3057[57.11]	3740[63.47]	1109[59.15]	311[58.57]
40-59 (male) / 20-39 (female)	608[11.36]	275[4.67]	61[3.25]	13[2.45]
60+ (male) / 40+ (female)	496[9.27]	111[1.88]	11[0.59]	10[1.88]
Total energy intake kJ/day)	11021[4163]	9394[3026]	10654[3724]	15118[6722]
Protein g/day)	115.03[58.34]	99.70[31.66]	113.74[37.07]	158.72[68.17]
Fat (g/day)	101.42[48.08]	82.99[30.04]	89.67[35.12]	120.55[59.28]
Carbohydrates (g/day)	269.27[109.97]	251.92[95.92]	305.97[121.15]	459.56[230.14]
Fibre (g/day)	30.97[14.23]	30.93[11.88]	36.43[12.94]	47.98[24.20]
Sodium (mg/day)	3701[1806]	3210[1074]	3518[1218]	4684[2082]
Potassium (mg/day)	3790[1613]	3776[1485]	4966[2187]	8053[4698]
Calcium (mg/day)	728.60[324.76]	824.43[285.63]	1153.22[355.46]	1916.96[741.95]
Phosphorus (mg/day)	1767[811]	1712[621]	2220[880]	3599[1819]
Calcium supplement use (n[%])	122[2.2]	165[2.7]	54[2.8]	27[5.0]
Dairy product intake (serves/week)				
0	2395[44.1]	1310[22.0]	79[4.1]	1[0.1]
1	1448[26.7]	1650[27.7]	393[20.7]	40[7.4]
2	1005[18.5]	1680[28.2]	541[28.5]	113[21.0]
3+	573[10.5]	1301[21.9]	880[46.4]	384[71.3]
Type of milk used (n[%])				
Full cream	2739[50.5]	2517[42.3]	586[30.9]	163[30.3]
Reduced fat	1312[24.2]	2360[39.7]	963[50.8]	252[46.8]
Skim	362[6.68]	698[11.7]	302[15.9]	106[19.7]
Soy	204[3.76]	88[1.4]	12[0.6]	4[0.7]
I don't use milk	804[14.8]	278[4.6]	30[1.5]	13[2.4]
Vegetable intake (serves/day)				
0-3	1101[20.3]	1185[19.9]	271[14.3]	62[11.5]
4--5	2014[37.1]	2025[34.1]	570[30.1]	145[26.9]
6--7	1331[24.55]	1584[26.7]	513[27.1]	132[24.5]
7+	975[17.9]	1147[19.3]	539[28.4]	199[36.9]
Fruit intake (serves/day)				

0-2	1306[24.1]	1140[19.2]	231[12.2]	68[12.6]
2--3	2070[38.2]	2428[40.8]	660[34.8]	161[29.9]
4--6	1098[20.2]	1423[23.9]	550[29.0]	145[26.9]
6+	947[17.4]	950[15.9]	452[23.8]	164[30.4]
Fish intake (serves/day)				
0-1	1014[18.7]	1247[20.9]	386[20.3]	107[19.8]
1-1.5	1260[23.2]	1445[24.3]	429[22.6]	107[19.8]
1.5-2.5	1777[32.7]	2012[33.8]	651[34.3]	160[29.7]
2.5+	1370[25.2]	1237[20.8]	427[22.5]	164[30.4]
Physical activity score (n[%])				
0	1442[26.6]	1306[21.9]	338[17.8]	93[17.3]
1-3	1076[19.8]	1104[18.5]	307[16.2]	77[14.3]
4-5	1770[32.6]	1925[32.4]	643[33.9]	174[32.3]
6+	1133[20.9]	1606[27.0]	605[31.9]	194[36.0]
Socioeconomic disadvantage (n[%])				
1 (most)	1032[19.1]	960[16.2]	285[15.2]	87[16.4]
2	1221[22.6]	1193[20.1]	326[17.4]	92[17.3]
3	915[16.9]	951[16.1]	299[15.9]	74[13.9]
4	948[17.5]	1135[19.2]	377[20.1]	110[20.7]
5 (least)	1279[23.7]	1669[28.2]	584[31.2]	167[31.5]
Educational level attained (n[%])				
Never attended school	10[0.1]	24[0.4]	0[0.0]	0[0.0]
Some primary school	359[6.6]	279[4.7]	51[2.6]	13[2.4]
Completed primary school	905[16.6]	620[10.4]	167[8.8]	45[8.3]
Some high/technical school	1795[33.1]	1731[29.1]	515[27.2]	136[25.2]
Completed high/technical school	542[10.0]	705[11.8]	211[11.1]	58[10.7]
Other qualification	532[9.8]	568[9.5]	173[9.1]	54[10.0]
Some study tertiary degree/diploma	190[3.5]	248[4.1]	92[9.1]	39[7.2]
Completed tertiary degree/diploma	1088[20.1]	1766[29.7]	684[36.1]	193[35.8]

Supplementary Table 2. Clinical, nutritional and demographic information of women in the cohort

Calcium intake quartile (mg/day)	<600	600-999	1000-1399	≥1400
n	2547	9602	6410	2275
Adjusted calcium intake (mg/day)	429.65[195.73]	810.40[109.96]	1164.26[110.28]	1724.66[405.09]
Ethnicity (n[%])				
Anglo-Celtic	1697[66.6]	7054[73.5]	5404[84.3]	1972[86.7]
Southern European	850[33.4]	2548[26.5]	1006[15.7]	303[13.3]
Age (years)	54.8[8.3]	54.3[8.4]	54.5[8.4]	54.9[8.5]
Height (cm)	158.82[6.75]	159.58[6.67]	160.53[6.5]	160.88[6.77]
Weight (kg)	68.6[12.8]	68.3[12.3]	67.4[11.7]	66.9[11.8]
BMI (kg/m^2)	27.2[5.1]	26.8[4.9]	26.2[4.5]	25.9[4.6]
WC (cm)	81.47[12.21]	80.10[11.69]	78.62[11.12]	77.68[10.98]
SBP (mmHg)	137.00[20.22]	134.52[19.27]	133.54[19.02]	134.49[18.59]
DBP (mmHg)	74.21[11.13]	73.11[10.94]	72.12[10.81]	71.50[10.82]
HTN (n[%])	215[8.4]	646[6.7]	357[5.5]	115[5.0]
Ever smoked – yes/no (n[%])	764[30.0]	2883[30.0]	1965[30.7]	729[32.0]
Smoker (n[%])				
Never	1793[70.0]	6719[69.9]	4445[69.3]	1546[67.9]
Former	299[11.7]	917[9.5]	460[7.1]	178[7.8]
Current	465[18.2]	1966[20.4]	1505[23.4]	551[24.2]
Alcohol intake (g/day)				
0	1380[54.57]	4942[51.72]	3385[53.06]	1273[56.25]
1-39 (male) / 1-19 (female)	815[32.23]	3698[38.70]	2533[39.71]	851[37.60]
40-59 (male) / 20-39 (female)	241[9.53]	760[7.95]	402[6.3]	119[5.26]
60+ (male) / 40+ (female)	93[3.68]	155[1.62]	59[0.92]	20[0.88]
Total energy intake kJ/day)	9594[3937]	7703[2640]	8460[2770]	11235[4910]
Protein g/day)	95.80[46.17]	71.18[27.48]	72.48[27.41]	91.17[44.54]
Fat (g/day)	101.54[52.11]	83.33[28.92]	93.48[29.00]	124.23[52.01]
Carbohydrates (g/day)	242.84[109.93]	206.30[81.92]	240.66[90.24]	336.15[165.67]
Fibre (g/day)	30.51[15.11]	27.14[10.92]	31.42[10.81]	39.04[17.11]
Sodium (mg/day)	3328[1666]	2700[974]	2884[955]	3597[1674]
Potassium (mg/day)	3525[1759]	3227[1305]	4071[1601]	6146[3349]
Calcium (mg/day)	641.49[308.52]	686.81[248.26]	958.73[269.85]	1535.71[580.50]
Phosphorus (mg/day)	1544[758]	1396[526]	1769[631]	2712[1308]
Calcium supplement use (n[%])	371[14.5]	1455[15.1]	1115[17.3]	456[20.0]
Dairy product intake (serves/week)				
0	1277[50.1]	2833[29.5]	494[7.7]	4[0.1]
1	616[24.1]	2695[28.1]	1486[23.1]	221[9.7]
2	443[17.3]	2568[26.7]	2030[31.6]	526[23.1]
3+	211[8.2]	1506[15.6]	2400[37.4]	1524[66.9]
Type of milk used (n[%])				
Full cream	1045[41.0]	3485[36.2]	1268[19.7]	365[16.0]
Reduced fat	601[23.6]	3609[37.5]	3219[50.2]	1133[49.8]
Skim	238[9.3]	1550[16.1]	1732[27.0]	727[31.9]
Soy	191[7.5]	309[3.2]	74[1.1]	18[0.7]
I don't use milk	472[18.5]	649[6.7]	117[1.8]	32[1.4]
Vegetable intake (serves/day)				
0-3	311[12.2]	1085[11.3]	449[7.0]	106[4.6]
4--5	765[30.0]	2966[30.8]	1547[24.1]	421[18.5]
6--7	698[27.4]	2963[30.8]	2125[33.1]	637[28.0]
7+	773[30.3]	2588[26.9]	2289[35.7]	1111[48.8]
Fruit intake (serves/day)				

0-2	375[14.7]	1397[14.4]	500[7.8]	123[5.4]
2--3	826[32.4]	3444[35.8]	2156[33.6]	600[26.3]
4--6	596[23.4]	2575[26.8]	1968[30.7]	705[30.9]
6+	750[29.4]	2186[22.7]	1786[27.8]	847[37.2]
Fish intake (serves/day)				
0-1	492[19.3]	1978[20.6]	1115[17.3]	370[16.2]
1-1.5	590[23.1]	2341[24.3]	1492[23.3]	438[19.2]
1.5-2.5	836[32.8]	3277[34.1]	2200[34.3]	717[31.5]
2.5+	628[24.6]	2006[20.8]	1603[25.0]	750[32.9]
Physical activity score (n[%])				
0	738[28.9]	2322[24.1]	1148[17.9]	345[15.1]
1-3	596[23.4]	2150[22.3]	1321[20.61]	390[17.1]
4-5	843[33.1]	3370[35.1]	2384[37.1]	903[39.6]
6+	370[14.5]	1760[18.3]	1557[24.2]	637[28.0]
Socioeconomic disadvantage (n[%])				
1 (most)	537[21.1]	1785[18.6]	1058[16.6]	365[16.2]
2	565[22.2]	2113[22.1]	1240[19.4]	407[18.0]
3	434[17.1]	1446[15.1]	1003[15.7]	317[14.0]
4	443[17.4]	1693[17.7]	1229[19.3]	447[19.8]
5 (least)	557[21.9]	2518[26.3]	1839[28.8]	723[32.0]
Educational level attained (n[%])				
Never attended school	10[0.1]	89[0.9]	37[0.5]	7[0.3]
Some primary school	359[6.6]	823[8.5]	284[4.4]	86[3.7]
Completed primary school	905[16.7]	1268[13.2]	536[8.3]	187[8.2]
Some high/technical school	1795[33.1]	4073[42.4]	2796[43.6]	996[43.7]
Completed high/technical school	542[10.0]	868[9.0]	650[10.1]	231[10.1]
Other qualification	532[9.8]	508[5.2]	366[5.7]	143[6.2]
Some study tertiary degree/diploma	190[3.5]	248[2.5]	222[3.4]	77[3.3]
Completed tertiary degree/diploma	1088[20.0]	1725[17.9]	1519[23.7]	548[24.1]

Supplementary table 3. Mortality rates and hazard ratios (HR) in women

		<600	600-999	1000-1399	1400+
	Calcium intake (mg/day)	<600	600-999	1000-1399	1400+
	Number at entry	2547	9602	6410	2275
	Number at exit	2151	8300	5620	1975
	Follow up time (years)	12.83[2.72]	12.52[6.81]	12.23[2.69]	12.06[2.60]
	Person-years at risk	21054.4	81411.4	57142.8	19723.8
	Person-years at risk per 1000	21.0544	81.4114	57.1428	19.7238
All-cause mortality	Number of deaths	396	1302	790	300
	Rate per 1000	6.41[5.41, 7.59]	6.17[5.66, 6.74]	6.19[5.58, 6.87]	5.72[4.76, 6.88]
	Crude HR [95% CI]	0.93[0.77, 1.13]	Ref	1.02[0.89, 1.17]	1.03[0.84, 1.26]
	Adjusted HR [95% CI]	0.96[0.79, 1.18]	Ref	0.94[0.81, 1.09]	0.85[0.66, 1.10]
Cardiovascular mortality	Number of deaths	102	295	175	76
	Rate per 1000	1.66[1.19, 2.31]	1.40[1.16, 1.68]	1.66[1.35, 2.03]	1.87[1.35, 2.58]
	Crude HR [95% CI]	1.06[0.72, 1.55]	Ref	1.20[0.92, 1.58]	**1.49[1.02, 2.16]**
	Adjusted HR [95% CI]	1.20[0.80, 1.78]	Ref	1.06[0.79, 1.41]	1.11[0.69, 1.81]
Myocardial infarction	Number of deaths	25	62	35	18
	Rate per 1000	0.33[0.15, 0.69]	0.22[0.13, 0.35]	0.24[0.14, 0.41]	0.30[0.13, 0.67]
	Crude HR [95% CI]	1.35[0.56, 3.25]	Ref	1.12[0.56, 2.26]	1.54[0.61, 3.88]
	Adjusted HR [95% CI]	1.65[0.65, 4.18]	Ref	0.90[0.43, 1.91]	0.79[0.22, 2.76]

Supplementary table 4. Mortality rates and hazard ratios (HR) in men

	Calcium intake (mg/day)	<600	600-999	1000-1399	1400+
	Number at entry	5421	5928	1893	538
	Number at exit	4140	4726	1528	423
	Follow up time (years)	12.90[3.04]	12.51[2.93]	12.36[2.96]	12.32[2.85]
	Person-years at risk	44043.3	49919.6	16300.0	4694.1
	Person-years at risk per 1000	44.0433	49.9196	16.3	4.6941
All-cause mortality	Number of deaths	1281	1203	365	115
	Rate per 1000	10.28[9.38, 11.27]	8.95[8.16, 9.82]	9.57[8.18, 11.19]	10.65[8.07, 14.05]
	Crude hazard ratio [95% CI]	0.98[0.86, 1.12]	Ref	1.09[0.90, 1.30]	**1.34[1.00, 1.79]**
	Adjusted hazard ratio [95% CI]	1.01[0.88, 1.15]	Ref	1.09[0.90, 1.33]	**1.42[1.02, 1.99]**
Cardiovascular mortality	Number of deaths	319	295	92	34
	Rate per 1000	2.04[1.66, 2.51]	2.02[1.66, 2.45]	2.39[1.74, 3.27]	2.76[1.60, 4.76]
	Crude hazard ratio [95% CI]	0.87[0.65, 1.16]	Ref	1.19[0.82, 1.73]	1.51[0.84, 2.69]
	Adjusted hazard ratio [95% CI]	0.86[0.64, 1.16]	Ref	1.25[0.84, 1.86]	1.83[0.94, 3.55]
Myocardial infarction	Number of deaths	76	77	20	8
	Rate per 1000	0.43[0.27, 0.67]	0.40[0.25, 0.62]	0.61[0.33, 1.14]	0.42[0.10, 1.70]
	Crude hazard ratio [95% CI]	0.93[0.50, 1.75]	Ref	1.53[0.72, 3.28]	1.09[0.25, 4.70]
	Adjusted hazard ratio [95% CI]	0.95[0.49, 1.87]	Ref	1.54[0.66, 3.59]	1.84[0.34, 9.91]

Supplementary table 5. Mortality rates and hazard ratios (HR) relative to the second group in women excluding outliers with calcium intake greater than three SD above the mean

		G1	G2	G3	G4
	Calcium intake (mg/day)	<600	600-999	1000-1399	1400+
	Number at entry	2547	9572	6399	2019
	Number at exit	2151	8275	5609	1751
	Follow up time (years)	12.83[2.72]	12.52[2.68]	12.23[2.69]	12.08[2.63]
	Person-years at risk	21054.4	81411.4	57142.8	17734.8
	Person-years at risk per 1000	21.0544	81.4114	57.1428	17.7348
All-cause mortality	Number of deaths	396	1297	790	268
	Rate per 1000	6.41[5.41, 7.59]	6.17[5.66, 6.74]	6.19[5.58, 6.87]	5.97[4.94, 7.23]
	Crude HR [95% CI]	0.93[0.77, 1.13]	Ref	0.99[0.86, 1.14]	1.03[0.83, 1.27]
	Adjusted HR [95% CI]	0.96[0.79, 1.17]	Ref	0.94[0.81, 1.09]	0.86[0.66, 1.11]
Cardiovascular mortality	Number of deaths	102	295	175	71
	Rate per 1000	1.66[1.19, 2.31]	1.40[1.16, 1.68]]	1.66[1.35, 2.03]	1.97[1.41, 2.74]
	Crude HR [95% CI]	1.06[0.72, 1.55]	Ref	1.20[0.92, 1.58]	1.50[1.02, 2.19]
	Adjusted HR [95% CI]	1.02[0.80, 1.79]	Ref	1.06[0.79, 1.42]	1.13[0.69, 1.85]
Myocardial infarction	Number of deaths	25	62	35	16
	Rate per 1000	0.30[0.17, 0.51]	0.22[0.12, 0.42]	0.15[0.07, 0.31]	0.31[0.18, 0.53]
	Crude HR [95% CI]	1.35[0.56, 3.25]	Ref	1.12[0.56, 2.26]	1.36[0.50, 3.67]
	Adjusted HR [95% CI]	1.69[0.66, 4.28]	Ref	0.84[0.39, 1.78]	0.58[0.15, 2.22]

Supplementary table 6. Mortality rates and hazard ratios (HR) relative to the second group in men excluding outliers with calcium intake greater than three SD above the mean

	Calcium intake (mg/day)	<600	600-999	1000-1399	1400+
	Number at entry	5421	5929	1893	452
	Number at exit	4140	4726	1528	354
	Follow up time (years)	12.97[3.04]	12.50[2.93]	12.36[2.96]	12.25[2.81]
	Person-years at risk	44043.3	50029.1	16300	3984.3
	Person-years at risk per 1000	44.0433	50.0291	16.3	3.9843
All-cause mortality	Number of deaths	1281	1203	365	98
	Rate per 1000	10.28[9.38, 11.27]	8.93[8.14, 9.80]	9.57[8.18, 11.19]	11.04[8.21, 14.83]
	Crude HR [95% CI]	0.98[0.86, 1.13]	Ref	1.08[0.89, 1.30]	**1.38[1.00, 1.92]**
	Adjusted HR [95% CI]	1.00[0.87, 1.15]	Ref	1.10[1.08, 1.34]	**1.54[1.08, 2.19]**
Cardiovascular mortality	Number of deaths	319	295	92	31
	Rate per 1000	2.04[1.66, 2.51]	2.02[1.66, 2.45]	2.39[1.74, 3.27]	3.26[1.89, 5.61]
	Crude HR [95% CI]	0.87[0.65, 1.16]	Ref	1.19[0.82, 1.73]	**1.86[1.04, 3.32]**
	Adjusted HR [95% CI]	0.86[0.64, 1.16]	Ref	1.25[0.85, 1.87]	**2.25[1.16, 4.37]**
Myocardial infarction	Number of deaths	76	77	20	7
	Rate per 1000	0.43[0.27, 0.67]	0.40[0.25, 0.62]	0.61[0.33, 1.14]	0.50[0.12, 2.00]
	Crude HR [95% CI]	0.93[0.50, 1.75]	Ref	1.53[0.72, 3.28]	1.35[0.31, 5.79]
	Adjusted HR [95% CI]	0.96[0.49, 1.87]	Ref	1.54[0.66, 3.60]	2.12[0.39, 11.35]

Supplementary table 7: Adjusted hazard ratios (HR) in women according Swedish thresholds for calcium intake

SEIFA disadvantage		Energy adjusted calcium intake (mg/day)							
		<600 [n=2547]		600-999 [n=9602]		1000-1399 [n=6410]		≥1400 [n=2275]	
		n (events)	HR[95%CI]	n (events)	HR[95%CI]	n (events)	HR[95%CI]	n (events)	HR[95%CI]
All-cause mortality	Most (1)	537 (107)	1.38[0.85, 2.23]	1785 (279)	Ref	1058 (166)	0.82[0.56, 1.18]	365 (61)	1.66[0.89, 3.12]
	2	565 (96)	0.82[0.53, 1.26]	2113 (348)	Ref	1240 (166)	0.92[0.67, 1.27]	407 (60)	0.72[0.39, 1.31]
	3	434 (50)	1.26[0.70, 2.27]	1446 (191)	Ref	1003 (126)	1.23[0.84, 1.80]	317 (41)	0.69[0.30, 1.55]
	4	443 (66)	0.91[0.55, 1.50]	1963 (196)	Ref	1229 (145)	1.33[0.93, 1.92]	447 (55)	1.25[0.71, 2.21]
	5 (least)	557 (76)	0.92[0.60, 1.40]	2518 (280)	Ref	1839 (183)	0.74[0.55, 0.98]	723 (81)	**0.52[0.30, 0.90]**
Cardiovascular mortality	Most (1)	537 (30)	1.70[0.65, 4.45]	1785 (74)	Ref	1058 (35)	1.13[0.55, 2.30]	365 (14)	2.45[0.70, 8.57]
	2	565 (24)	1.37[0.58, 3.27]	2113 (78)	Ref	1240 (47)	1.32[0.69, 2.49]	407 (10)	0.61[0.17, 2.16]
	3	434 (13)	1.20[0.39, 3.64]	1446 (39)	Ref	1003 (23)	0.89[0.40, 1.99]	317 (9)	0.76[0.16, 3.54]
	4	443 (16)	1.24[0.48, 3.18]	1963 (40)	Ref	1229 (28)	1.10[0.51, 2.33]	447 (21)	1.87[0.66, 5.31]
	5 (least)	557 (19)	0.89[0.33, 2.36]	2518 (62)	Ref	1839 (40)	0.89[0.49, 1.59]	723 (21)	0.60[0.21, 1.67]

Supplementary table 8: Adjusted hazard ratios (HR) in men according Swedish thresholds for calcium intake

| | | Energy adjusted calcium intake (mg/day) | | | | | | | |
| | | <600 [n=5421] | | 600-999 [n=5941] | | 1000-1399 [n=1893] | | ≥1400 [n=538] | |
SEIFA		n (events)	HR[95%CI]	n (events)	HR[95%CI]	n (events)	HR[95%CI]	n (events)	HR[95%CI]
All-cause mortality	Most (1)	1032 (305)	1.10[0.79, 1.55]	960 (237)	Ref	285 (72)	0.90[0.54, 1.50]	87 (26)	1.93[0.80, 4.64
	2	1221 (318)	0.83[0.62, 1.10]	1193 (312)	Ref	326 (61)	0.98[0.61, 1.58]	92 (20)	1.37[0.51, 3.62]
	3	915 (210)	1.43[0.98, 2.08]	951 (178)	Ref	299 (60)	1.59[0.93, 2.70]	74 (14)	0.65[0.16, 2.63]
	4	948 (211)	1.00[0.70, 1.42]	1135 (209)	Ref	377 (72)	**1.86[1.16, 2.97]**	110 (17)	0.94[0.39, 2.30]
	5 (least)	1279 (232)	1.06[0.79, 1.43]	1669 (263)	Ref	584 (96)	0.76[0.52, 1.12]	167 (36)	**1.86[1.08, 3.21]**
Cardiovascular mortality	Most (1)	1032 (79)	0.62[0.33, 1.19]	960 (67)	Ref	285 (21)	0.66[0.24, 1.79]	87 (7)	2.13[0.37, 12.12]
	2	1221 (78)	0.86[0.46, 1.61]	1193 (72)	Ref	326 (9)	1.06[0.33, 3.38]	92 (7)	7.80[1.63, 37.31]
	3	915 (57)	1.51[0.60, 3.76]	951 (45)	Ref	299 (23)	4.92[1.77, 13.69]	74 (5)	6.94[0.83, 57.79]
	4	948 (46)	0.51[0.21, 1.21]	1135 (45)	Ref	377 (20)	5.26[1.73, 15.93]	110 (6)	3.57[0.54, 24.49]
	5 (least)	1279 (57)	0.99[0.50, 1.94]	1669 (63)	Ref	584 (20)	0.48[0.21, 1.09]	167 (9)	0.54[0.15, 1.89]

Supplementary table 9: Adjusted hazard ratios (HR) according Swedish thresholds for calcium intake

| | | | Energy adjusted calcium intake (mg/day) [n=women/men] | | | | | | | |
| | | | <600 [n=2547/5421] | | 600-999 [n=9602/5941] | | 1000-1399 [n=6410/1893] | | ≥1400 [n=2275/538] | |
		Calcium taken	n (events)	HR[95%CI]	n (events)	HR[95%CI]	n (events)	HR[95%CI]	n (events)	HR[95%CI]
Women	All-cause	No	2176 (349)	1.00[0.80, 1.24]	8147 (1104)	Ref	5295 (665)	0.94[0.80, 1.10]	1819 (227)	0.88[0.65, 1.19]
		Yes	371 (47)	1.02[0.59, 1.75]	1455 (198)	Ref	1115 (125)	0.98[0.67, 1.42]	456 (73)	0.64[0.36, 1.16]
	Cardiovascular	No	2176 (92)	1.19[0.77, 1.84]	8147 (242)	Ref	5295 (146)	1.05[0.77, 1.45]	1819 (53)	1.05[0.60, 1.84]
		Yes	371 (10)	1.65[0.54, 5.06]	1455 (54)	Ref	1115 (29)	1.05[0.46, 2.38]	456 (23)	1.31[0.43, 3.91]
Men	All-cause	No	5299 (1245)	0.99[0.86, 1.14]	5776 (1168)	Ref	1839 (353)	1.10[0.90, 1.34]	511 (112)	**1.44[1.02, 2.02]**
		Yes	122 (36)	2.81[0.51, 15.39]	165 (36)	Ref	54 (12)	0.29[0.01, 6.72]	27 (3)	6.25[0.14, 263.84]
	Cardiovascular	No	5299 (306)	0.80[0.59, 1.09]	5776 (290)	Ref	1839 (90)	1.28[0.86, 1.90]	511 (33)	1.87[0.94, 3.70]
		Yes	122 (13)	n/e	165 (6)	Ref	54 (3)	n/e	27 (1)	n/e

Chapter 5: Conclusions and Future Directions

Conclusions

This thesis has produced three main findings including (i) there appears to be a direct relationship between muscle and vascular disease; (ii) low bone density appears to have a direct relationship to cardiac health, influenced by the presence of aortic calcification and (iii) calcium and vitamin D have modest effects on vascular disease.

There are many potential explanatory reasons for these shared relationships. Most significantly, muscle, bone and vascular disease share a number of risk factors such as smoking, poor diet and lifestyle characteristics (e.g. low levels of physical activity) as well as co-morbidities including type 2 diabetes, human immunodeficiency virus infection, kidney disease and chronic obstructive pulmonary disease [47]. There are also a number of common pathological features including the role of inflammation, Wnt signalling pathways, macrophage infiltration to sites of injury and smooth muscle cell alterations that may contribute to the concomitant development of muscle loss, bone loss and vascular disease [35, 48]. However, despite these observations supporting *indirect* associations between muscle, bone and vascular disease there has been little *direct* clinical evidence to support these associations particularly in healthy populations. Thus, the scientific motivation for the thesis was to explore the musculoskeletal aspects of vascular disease.

In the second chapter, the association of components of muscle loss (muscle mass and strength measures) with vascular diseases was examined. Previous research has consistently revealed an inverse association between muscle mass and arterial stiffness though these studies were heterogeneous in many aspects. Therefore, it was unknown if these associations were relevant across the various populations and other sources of heterogeneity such as the method to quantify muscle mass. In simple terms, it was unknown if these associations were generalisable. In a meta-analysis compiling these data, it was demonstrated that the association was consistent including across various stratifications by sources of heterogeneity and thus we concluded that arterial stiffness is likely a feature of muscle loss [Chapter 2.2]. To put it another way, muscle loss is likely to have a negative impact on the vasculature. Muscle loss is a common feature alongside bone loss during ageing. The links between bone loss and vascular calcification are well established, however, no previous analysis explored the potential association between muscle mass and AAC. This is important because AAC is a robust indicator of vascular disease and this validates and extends our understanding from what was previously shown in the meta-analysis of muscle mass and arterial stiffness. In a cross-sectional analysis of healthy, older men and women, it was shown that individuals with the lowest amount of muscle mass relative to body mass index (thus the amount of muscle mass was normalised to body size) had the greatest likelihood of having both any and severe AAC [Chapter 2.3]. Importantly in older age, the loss in muscle strength appears to exceed the actual loss of muscle mass and thus muscle 'quality' may be a more

important construct in determining muscle health [33]. These aspects of muscle quality appear partly attributable to neuronal innervation which requires adequate vascular supply that may be disrupted by the presence of calcification in those supplying vessels. Muscle quality and its association with vascular disease including calcification is unknown. Most previous studies have examined physical function in relation to subclinical atherosclerosis; and other studies have sought to examine the association between frailty (definitions of which incorporates measures of muscle strength and quality) and vascular events and arterial stiffness as these outcomes are readily available either through registries or obtained in an office setting in the case of arterial stiffness. No study had directly investigated AAC and its association with neuromuscular function. Thus, in a cross-sectional and longitudinal study of healthy older women who participated in a randomised controlled trial of calcium intake on fracture outcomes; our data supports a view that having AAC predicts accelerated declines in handgrip strength but not mobility [Chapter 2.4]. This result was surprising but given what previous literature suggests [Chapter 1.2], it is actually unsurprising as in older age the effects of vascular disease on physical function appear to be more pronounced than the effects of physical function on vascular disease. Therefore, future studies are needed to test the hypothesis that poor physical function negatively affects the vasculature in middle-aged individuals. This would help clarify the potential the complex nature of the muscle function-vascular disease relationship throughout the life course.

Muscle has a close relationship with bone and vice-versa. The past few decades has firmly established links between bone loss and calcification [49]. The third chapter was dedicated to exploring less understood links between bone and the vasculature such as the cardiac consequences of having low bone density and if this relationship can be explained by the presence of aortic calcification. This is important as abnormal heart function has been demonstrated in individuals with low bone density but what role calcification has in this relationship is unclear [22, 50, 51]. In healthy older adults, it was demonstrated that lower BMD was associated with greater cardiac workload (estimated as the rate pressure product, a proxy measure for cardiomyocyte oxygen consumption) and this association was mediated (in part) by calcification [Chapter 3.2]. This association was more strongly evident in women than in men suggestive of a role for sex steroid hormones. What this chapter further highlighted was the potential to incorporate detection and quantification of AAC at the time of bone density assessment [4]. AAC can be visualised during lateral spine densitometry (usually undertaken to determine vertebral fractures). Thus, there is an opportunity to screen for both osteoporosis and cardiovascular disease in the same scan which would additionally mean there may also be a cost-benefit to the procedure. This is particularly relevant given our newly appreciated understanding that these diseases commonly co-occur during ageing and may go some way to influencing clinician decision making [52]. Interpretation of AAC scans requires additional expertise and reporting burden however, with the advent of machine learning in medicine this barrier may be overcome in the short to medium term.

The final chapter of this thesis investigated the effects of vitamin D and calcium (factors critical to musculoskeletal health and frontline public health practice for the maintenance of bone health) on cardiovascular disease prevalence and management. Two meta-analyses on the effect of vitamin D on measures of arterial stiffness [Chapter 4.2] and on inflammatory cytokines [Chapter 4.3] in patients with established cardiovascular disease. In both instances, vitamin D had modest effects on these outcomes. It was concluded that vitamin D may either be insufficient as a stand-alone intervention to produce clinically significant effects or that low vitamin D represents a consequence of these disease states and not the cause. In both instances, from a biological standpoint, vitamin D appears to be just a single element in a more complex underlying pathophysiology.

Controversy still remains regarding the cardiovascular safety of calcium intakes from dietary sources as there have been numerous trials demonstrating relative beneficial effects, but also harmful effects [53]. Of interest, a Swedish study showed a potentially adverse cardiovascular effect of high calcium intakes in women; whilst a similar study in Australia showed relative benefits in men and women combined. We investigated possible sex-specific effects of dietary calcium intake and, surprisingly, the previous results from the Swedish study could not be replicated in Australian individuals. Indeed, there instead appeared to be a modest beneficial effect of high dietary calcium in Australian women though not statistically significant [Chapter 4.4].

Though in men, it appeared that high dietary calcium intakes were associated with relative harms. Future studies are needed to directly examine potential reasons for these sex differences and given that in the Australian cohort examined in the study, approximately 25% were of non-Anglo-Celtic origin, there could be ethnic differences that help explain heterogeneity between previous studies. It is speculated that it may not be the ethnic origins of participants *per se* that drive risk differences but the overall lifestyle differences including sources of dietary calcium and total diet variety inherent in different ethnicities that explain the risk differences. It is also possible that differences in general metabolism or underlying and unquantified genetic differences explain risk discrepancies. Calcium (including both increasing intake dietary sources or supplemental sources in cases of poor dietary choices or lack of access to calcium rich food) is recommended for the prevention of osteoporosis at a community/public health level and there appears to be little to modest evidence to support discontinuation of current public health practices. Fortification of food (with calcium and/or vitamin D) may be an effective way to boost population level calcium intake and serum vitamin D and would circumvent potential adherence issues associated with supplementation and changing usual dietary patterns.

Future Directions

Several lines of inquiry have been established [see table below]. Chapter two provided evidence for a muscle-vascular axis and hypotheses generated from these studies warrant further investigation. As these studies were cross-sectional and

conducted in older Caucasian cohorts, future studies should be conducted in younger cohorts, include both men and women, non-Caucasians and be preferably free of disease. AAC may already be established in the cohorts investigated and thus a prospective study examining causes of AAC development would be the most informative as it will enable understanding of whether declines in muscle mass and quality do indeed precede AAC development and if low muscle mass and strength is predictive of vascular calcification. The question of whether muscle mass and strength influence vascular disease onset or progression can be answered from interventional studies. Exercise has known beneficial effects on bone mass, muscle mass and physical function, and observational studies have suggested that in those who exhibit high levels of physical activity develop less vascular disease [54] – therefore could there be an interventional/therapeutic benefit of exercise on calcification. In this setting we would be able to infer a direct shared biology if it can be shown that strategies that promote mass growth and strength gains (i.e. exercise) concomitantly promotes regression of AAC and reduction in arterial stiffness. That is to say - does the improvement in muscle mass or strength correlate with improvement in vascular disease markers? To this end, a current trial is underway [Australia New Zealand Clinical Trials Registry #ACTRN12616000563460, for which I have primary involvement] and the above research questions form secondary outcomes. Also, it would be informative to investigate if known cardio-protective interventions (such as medications) have benefit in promoting muscle mass and strength gains or maintenance throughout

older age. As alluded above, the natural history of a shared relationship between muscle and vascular disease is unknown and longitudinal studies are needed to determine if declines in mass/strength (seen with ageing) predict the development of AAC (also seen with ageing). In Chapter 2.3 muscle mass determined aortic calcification yet in Chapter 2.4 aortic calcification determined muscle strength. Thus, the nature of the relationship between physical function and aortic calcification in older adults needs to be determined. Evidence from Chapter 1.2 suggested that the effect of poor physical function on aortic calcification is more pronounced earlier in life and that in older age the effect of aortic calcification on physical function is more evident. No data as yet exists to answer this question.

Chapter three highlighted the need to investigate if menopause and sex steroid hormones potentially influence cardiac function helping to explain increasing cardiovascular risk in older age. This is especially relevant as men and women appear to have different risk profiles with respect to cardiovascular diseases in older age. Furthermore, given that heart diseases disproportionately affect men compared to women [55], it is interesting to note that disease burden of aortic calcification (which would increase cardiovascular risk) was more pronounced in women than in men in this study. Further to comments above, dual screening for calcification at the time of bone density assessment may be a cost-effective way to identify women at increased cardiovascular disease risk given that health attention in women

immediately post the menopause is focussed on skeletal health. Data from this thesis suggests skeletal health may be inextricably linked to vascular health .

Chapter four investigated potential beneficial effects of vitamin D supplementation on inflammation and on arterial function. Although limited benefit was seen in these contexts, it is uncertain if these modest effects of vitamin D in disease outcomes could be augmented by combining the intervention with an adjunctive therapy. For instance, obesity is common in older age and given that vitamin D is a fat-soluble steroid hormone, it is known to become sequestered in adipose tissue limiting bioavailability. Weight loss has been shown to improve vitamin D levels as well as have independent effects on inflammation and arterial stiffness [56]. Thus, there could be benefit in combining vitamin D with weight loss therapy such as exercise to improve vascular disease markers.

Also examined was the cardiovascular safety of calcium intake. Given highly publicised studies demonstrating potential cardiovascular effects of calcium supplements, a clinician may be prudent to advise adequate calcium intake from the diet instead of supplements for the maintenance of healthy bones [46, 57]. This thesis determined potential adverse effects (in men) of high dietary calcium and thus, it may be further prudent to investigate potential adverse effects of medications used to promote bone health. The most commonly prescribed medications are the bisphosphonates which promote osteoclast apoptosis and thus limit bone loss.

Bisphosphonates have been previously linked to an increase in cardiovascular risk, but causality remains controversial [58–60]. In 2013, Grove *et al.* linked the use of bisphosphonates to incident heart failure in a study using Danish health data. In a sample of predominantly older (mean age 71 years) women (85%), the use of any bisphosphonate was associated with an approximate 40% increased risk of incident heart failure relative to those who were bisphosphonate naïve [60]. There was no gender difference in risk, though the risk profile differed amongst nitrogen-containing bisphosphonates (e.g. alendronate) and non-nitrogen-containing bisphosphonates (e.g. etidronate). Another study determined a protective effect of bisphosphonate use. In a cohort of hip fracture patients in Hong Kong, bisphosphonate use was associated with a 67% reduced risk of one-year cardiovascular mortality and this beneficial effect persisted in the long-term (risk reduction of 18% at five years and 17% at 10 years) [61]. These studies were limited in their investigation of prognostic factors for heart failure as data was captured from national health register records rather than clinical records such as patient notes. Therefore, taking into consideration important risk factors such as renal function, vitamin D status, body mass index, BMD and co-medications; we may be able to better characterise the risk of heart failure from bisphosphonate therapy. Furthermore, we may also be able to better identify specific patient sub-sets who may be at elevated risk levels.

To this end, I was very fortunate to secure an extremal grant to undertake my first 'post-doctoral' position as a Guest Researcher at Syydansk Universitet (Odense, Denmark) investigating the cardiovascular safety of bisphosphonates. This project represents intellectual and professional progression from my current work as the project topic will be immediately translatable into clinical practice or at the least be able to inform clinical practice, whilst the majority of studies conducted in this thesis are still in the hypothesis generating side of the scientific continuum.

The current project is unique in that it is designed to (1) establish if osteoporosis drug use increases the risk of cardiovascular disease and (2) identify the characteristics of the patients who develop cardiovascular disease subsequent to osteoporosis drug treatment initiation and in this way highlight these individuals who may need to be recommended other treatments. Thus, this project paves the way for future studies investigating direct effects of osteoporosis medications on heart tissue. These potential studies are possible in *in vivo* (for example echocardiography) and *in vitro* (effect of osteoporosis drugs on cultured cardiac myocytes) settings or in animal models (examine *ex vivo* the heart and other affected organs in aged mouse models of heart failure treated with osteoporosis medications). These projects will form the basis of future studies I intend to plan through my academic network. Specifically, this thesis coincides with my active pursuit of collaborators of a project to characterise the effect of (or association of) AAC on cardiac structure and function as determined by echocardiography and electrocardiography. This research would

be the first to demonstrate associations aortic calcification with altered haemodynamics. Future studies would ideally link these data to cardiovascular outcomes from registries which would thus robustly describe a mechanism to support the observation of increased cardiovascular mortality in individuals with osteoporosis and osteopenia.

Future Research Questions

Research questions addressed in this thesis	Key findings	Relevant future research questions
What is the association of muscle mass with arterial stiffness?	Muscle mass explained approximately 18% of the variance in pulse wave velocity and approximately 15% in healthy individuals. Vascular site, muscle region, imaging modality or arterial stiffness device used did not significantly affect the outcome.	Does improving muscle mass through a direct intervention (for example resistance training exercise) result in improvements in arterial stiffness?
What is the association of muscle mass with abdominal aortic calcification?	Older healthy individuals in the lowest tertile of BMI-adjusted appendicular lean mass had an approximate two-and-a-half-fold increased likelihood of having abdominal aortic calcification.	Does improving muscle mass through a direct intervention (for example resistance training exercise) result in improvements in aortic calcification?
Does the presence of abdominal aortic calcification have an impact on physical function?	Older healthy women had an approximate 18% standard deviation greater decline in handgrip strength over five years.	Does a reduction in aortic calcification lead to improved physical function? Given aortic calcification is a marker of established cardiovascular disease, do cardio-protective medications have an effect on aortic calcification and physical function?
What is the association of bone density on cardiac function?	In healthy older adults, greater hip bone mineral density was associated with a lower rate pressure product and the presence of aortic calcification mediated 13.5% of this relationship. In women, this effect was more pronounced, with aortic calcification mediating 21% of this relationship.	What role do sex-steroid hormones play in the association between bone density and cardiac function? Will reporting of aortic calcification at the time of bone density screening lead to improved cardiovascular outcomes in women and is it cost-effective?

What is the effect of vitamin D supplementation on arterial stiffness?	Vitamin D supplementation (ranging from 1000-5700IU oral daily) in adults produced non-significant reductions in pulse wave velocity and augmentation index compared to treated individuals. Dosing, length of study, chronic kidney disease, studies involving specific patient groups and older age did not alter outcomes.	Can the effect of vitamin D be augmented by an adjunctive therapy?
What is the effect of vitamin D supplementation on inflammation in heart disease?	Vitamin D supplementation in older adults (predominantly men) with clinically diagnosed heart failure lead to significant reduction in serum level of the inflammatory marker TNF-α compared to placebo treated patients. There was no statistically significant effect on the other markers CRP, IL-6 or IL-10	Does vitamin D supplementation have immunomodulatory effects in other cardiovascular disease settings? Is low vitamin D a cause or consequence of heart failure?
What is the association of calcium intake on mortality outcomes?	In healthy middle-aged Australian adults, energy-adjusted dietary calcium intakes above 1400mg/day was associated with an approximate 42% increased all-cause mortality risk in men but a non-significant reduction in risk in women.	Does fortifying food with minerals reduce micronutrient deficiencies and does this lead to improve disease and mortality outcomes? What factor contributes to the sex-specific effect of calcium intake on mortality? Given the findings were in contradiction to a highly publicised study in Swedish women, what role does overall lifestyle and source of calcium have in explaining risk differences amongst ethnicities?

In summary, this programme of research has demonstrated relationships between the musculoskeletal system and vascular disease and further may have important cardiovascular effects. Given this, musculoskeletal diseases deserve more scientific, public and clinical attention.

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Appendices: other publications produced during candidature

Appendix 1:

Rodriguez AJ, Ebeling P (2018) At what price increased

mortality risk? Osteoporos Int. doi.org/10.1007/s00198-018-

4550-5

LETTER

CrossMark

At what price decreased mortality risk?

Response to 'Were VCF patients at higher risk of mortality following the 2009 publication of the vertebroplasty "sham" trials?'

A.J. Rodriguez[1] · P.R. Ebeling[1,2]

Dear Editor,

There is continued interest in the future of vertebral augmentation procedures in clinical practice following the highly publicised 'sham' trials in 2009 [1, 2]. Because of this, the popularity of vertebral augmentation has been in decline, despite evidence that vertebral augmentation increases survival [3]. Procedure uptake and survival were investigated by Ong et al. in their study of Medicare data on mortality outcomes following these procedures [4]. Ten-year mortality risk was lower in those undergoing percutaneous vertebroplasty (VP) and balloon kyphoplasty (BKP) compared to non-surgical management (NSM).

This finding, whilst supportive of a role for vertebral augmentation in clinical practice, may be biased by underlying socioeconomic factors that may ultimately govern decisions about opting for surgical or non-surgical management. Given the lack of information regarding these factors (owing to limited data richness from Medicare data), we would be interested in the author's comments on how costs of treatments potentially influenced the outcomes noted. Is it quite possible that opting for surgical over non-surgical management may be a proxy for wealth, access to certain services and healthcare utilisation more generally? In support of this hypothesis, patients with lower socioeconomic status as well as those from southern states were at an increased mortality risk compared to those of higher socioeconomic status. Also, patients from other presumably wealthier regions (e.g. north-east states) had increased survival. A previous study

indicated that use of domestic help following hip fractures had socioeconomic disparities supportive of a view that factors that promote beneficial outcomes may be accessed by those most able to afford them [5].

It is alarming though that reductions in mortality risks in the post-2009 (sham trials) era were attenuated. We speculate that the reduction in popularity of these procedures and subsequent increases in costs made these procedures unavailable to those who may not have the means to support themselves adequately post-surgery [6]. It was noted that length of stay in hospital was shorter for patients opting for NSM and that discharge to home was higher in these patients (avoiding in-hospital costs?). As Ong et al. noted 'home discharge rates appear to reflect a shifting of the NSM patients from inpatient to other facilities, and do not reflect recovery of the patients'. Discharge to care facilities was higher amongst BKP and VP patients, where the ratio of patients discharged to home relative to NSM was 2.27 (2.20, 2.35) and 1.86 (1.80, 1.93) for BKP and VP respectively. These data again are potentially reflective of greater healthcare utilisation amongst those more able to afford care.

Overall, Ong et al. demonstrate that surgical augmentation of vertebrae to treat VCF may have a future in clinical practice. Subsequent trials have demonstrated what *type* of patient is most likely to benefit from such procedures based mostly on clinical symptoms prior to surgery [7]. Additional attention needs to be paid to other patient factors such as ability to access services that contribute to treatment success in the short and long terms. Understanding of these factors may result in more favourable outcomes which may promote procedure uptake.

Compliance with ethical standards

Conflicts of interest None.

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Appendix 2:

Rodriguez AJ*, Fink H*, Mirigian L, et al (2017) "Pain, quality of life and safety outcomes of kyphoplasty for vertebral compression fractures: report of a task force of the American Society for Bone and Mineral Research". J Bone Miner Res. doi: 10.1002/jbmr.3170

Pain, Quality of Life, and Safety Outcomes of Kyphoplasty for Vertebral Compression Fractures: Report of a Task Force of the American Society for Bone and Mineral Research

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ABSTRACT

The relative efficacy and harms of balloon kyphoplasty (BK) for treating vertebral compression fractures (VCF) are uncertain. We searched multiple electronic databases to March 2016 for randomized and quasi-randomized controlled trials comparing BK with control treatment (nonsurgical management [NSM], percutaneous vertebroplasty [PV], KIVA VCF treatment system [Benvenue Medical, Inc., Santa Clara, CA, USA], vertebral body stenting, or other) in adults with VCF. Outcomes included back pain, back disability, quality of life, new VCF, and adverse events (AEs). One reviewer extracted data, a second checked accuracy, and two rated risk of bias (ROB). Mean differences and 95% confidence intervals (CIs) were calculated using inverse-variance models. Risk ratios of new VCF and AE were calculated using Mantel-Haenszel models. Ten unique trials enrolled 1837 participants (age range, 61 to 76 years; 74% female), all rated as having high or uncertain ROB. Versus NSM, BK was associated with greater reductions in pain, back-related disability, and better quality of life (k = 1 trial) that appeared to lessen over time, but were less than minimally clinically important differences. Risk of new VCF at 3 and 12 months was not significantly different (k = 2 trials). Risk of any AE was increased at 1 month (RR = 1.73; 95% CI, 1.36 to 2.21). There were no significant differences between BK and PV in back pain, back disability, quality of life, risk of new VCF, or any AE (k = 1 to 3 trials). Limitations included lack of a BK versus sham comparison, availability of only one RCT of BK versus NSM, and lack of study blinding. Individuals with painful VCF experienced symptomatic improvement compared with baseline with all interventions. The clinical importance of the greater improvements with BK versus NSM is unclear, may be due to placebo effect, and may not counterbalance short-term AE risks. Outcomes appeared similar between BK and other surgical interventions. Well-conducted randomized trials comparing BK with sham would help resolve remaining uncertainty about the relative benefits and harms of BK. © 2017 American Society for Bone and Mineral Research.

KEY WORDS: KYPHOPLASTY; VERTEBRAL COMPRESSION FRACTURE; OSTEOPOROSIS; PAIN; QUALITY OF LIFE; AGING

Introduction

In the United States, vertebral compression fractures (VCFs) account for nearly one-half of the approximately 1.5 million osteoporotic fractures every year.[1] VCFs can cause acute and chronic pain, physical impairment and disability, adversely impact quality of life, and are associated with an increased risk of future vertebral and nonvertebral fractures.[2]

Medical treatments of VCF may reduce pain,[3] but many patients still experience severe and sometimes long-lasting symptoms. Alternatively, several "vertebral augmentation" procedures have been developed to treat patients with symptomatic VCF. The simplest of these is percutaneous vertebroplasty (PV), in which bone cement is percutaneously injected inside the fractured vertebral body. Balloon kyphoplasty (BK) involves inflation of a balloon inside the fractured vertebral body and balloon removal before injection of bone cement. With vertebral body stenting (VBS), balloon inflation and removal is followed by insertion of an expandable scaffold before injection of bone cement.[4] With the KIVA® VCF treatment system (Benvenue Medical, Inc., Santa Clara, CA, USA), a nesting, vertically oriented, cylindrical column is inserted over a coil into the fractured vertebral body before injection of bone cement.[5]

Vertebral augmentation procedures may reduce pain and back-related disability by restoring vertebral height and stabilizing fractured vertebrae, but risks may include cement leakage associated nerve-root injury, rarely symptomatic pulmonary embolism, and a possible increase in frequency of subsequent vertebral fractures attributable to procedure-related alterations in spine biomechanics.[6] Of approximately 300,000 inpatient vertebral augmentation procedures performed in the United States between 2005 and 2010, 73% were BK and 27% were PV.[7]

Given that BK is the most commonly performed vertebral augmentation procedure, we undertook this systematic review to compare its efficacy, relative efficacy, and harms versus other treatments for VCF in middle-aged and older adults, including versus nonsurgical management (NSM), sham control, PV, and other vertebral augmentation techniques.

Methods

Data sources

We followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.[8] We searched MEDLINE (from 1946), Cochrane Library, EMBASE, CINAHL, Web of Science, Clinicaltrials.gov, and the World Health Organization International Clinical Trials Registry Platform Search Portal databases to November 2014 using the title/abstract search terms: [("kyphoplasty") AND ("randomized" OR "controlled") AND NOT "malignancy" AND NOT "review"] with no language restriction. We updated this search in March 2016.

Study selection and inclusion criteria

We included randomized controlled trials (RCTs) and quasi-randomized trials that enrolled adults aged ≥40 years with nontraumatic vertebral fractures and compared BK versus any treatment for at least one validated measure of pain, disability, physical function, health-related quality of life, participant-reported treatment success, balance, falls, posture, bone mineral density (BMD), incident clinical or radiographic vertebral fractures, or adverse events (AEs). Two reviewers independently examined titles, abstracts, and full articles for eligibility and resolved discrepancies by discussion and consensus.

Data extraction and risk of bias assessment

Two reviewers independently extracted data on study design, participant characteristics, intervention characteristics, outcomes and adverse events, and an additional reviewer checked accuracy.

Studies were evaluated for risk of bias using the Cochrane Collaboration Risk of Bias Tool (v5.1.0; The Cochrane Collaboration, London, UK; http://www.cochrane.org/), with potential sources of bias including random sequence generation, allocation concealment, blinding of participants and study personnel, blinding of outcome assessments, and completeness of reported outcomes data.[9] For each domain, risk of bias was rated as high, low, or unclear by two independent investigators and agreed to in a consensus meeting. When there were multiple reports for a single study, information from all reports was used to rate risk of bias for each domain for that study. Risk of bias was summarized for individual studies as low (low risk of bias for all domains), unclear (unclear risk of bias for ≥1 domain) or high (high risk of bias for ≥1 domain).

Data analysis

For continuous outcomes, mean differences (MD) and 95% confidence intervals (CIs) were calculated using inverse-variance models. For dichotomous outcomes, risk ratios (RR) and 95% CIs were calculated using Mantel-Haenszel models. Statistical heterogeneity was determined by the I^2 statistic, applying random-effects models when $I^2 > 50\%$.[9] When there were multiple reports of a single study, the most recent publication was used as the primary data source and the other reports were used to provide supplemental information.

Results

Literature search

Together, the initial and updated database searches yielded 2460 unique references. We excluded 2406 during title and abstract review and 40 during full-text review, leaving 14 reports of 10 unique studies that met eligibility criteria and were included for analysis[4,5,10–21] (Fig. 1). Characteristics of included studies are described in Table 1.

Risk of bias

Among the 10 unique eligible studies, the most common source of bias was lack of blinding, followed by inadequate or uncertain concealment of treatment allocation, and incomplete reporting of outcomes (Table 2), though it was not possible to mask assessors of radiographic outcomes to the vertebral cement in participants assigned to BK or other vertebral augmentation procedures. No trials were rated as having low risk of bias, two were rated as having unclear risk of bias,[5,14,15] and eight were rated as having high risk of bias.[4,10–13,16–21]

Kyphoplasty versus placebo or sham

We identified no eligible trials that compared BK versus a sham BK procedure.
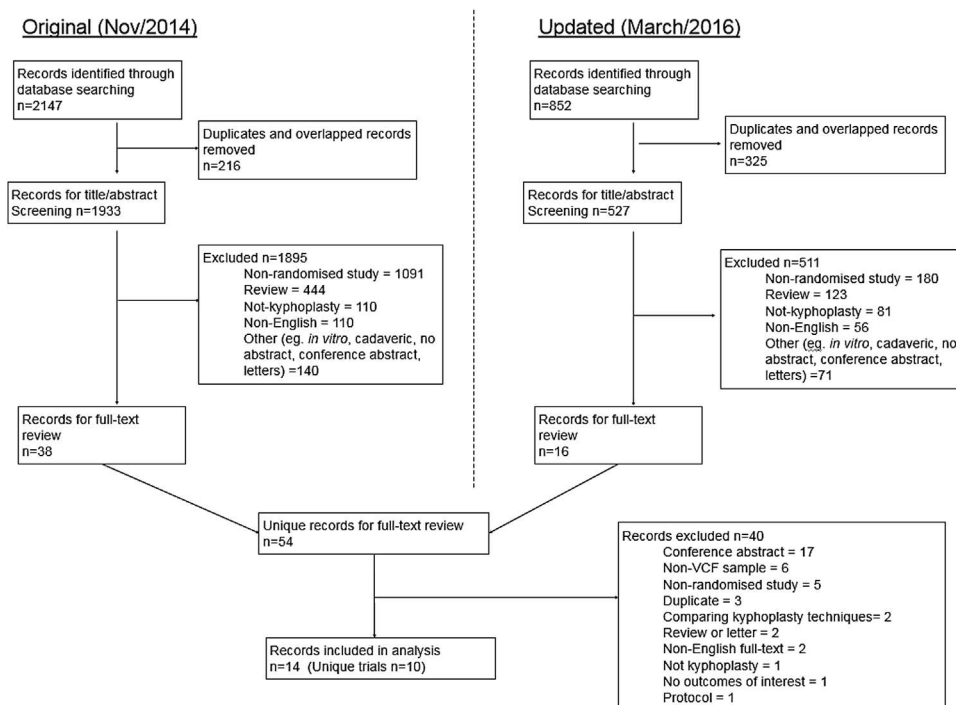
Fig. 1. Study selection flow diagram.

Kyphoplasty versus nonsurgical management

Study characteristics

Five reports met eligibility criteria, four of which were duplicate publications from the FREE trial,[10,11,17,20] so that there were two unique trials. Both were rated as having high risk of bias. The FREE trial ($n = 300$) used computer-generated permuted block randomization, after which participants and study staff were unblinded. Follow-up was 24 months. Yi and colleagues' trial ($n = 200$)[21] stated that participants were randomized, but also that treatment assignment was blindly chosen by a single surgeon. Outcome assessors but not participants were blinded to treatment assignment and follow-up was 48 months (Table 2).

Patient characteristics

In the FREE trial, mean age was 72.2 years, and 77% of participants were female.[10,11,17,20] Qualifying vertebral fractures were ≤3 months old (mean 6 weeks) and most commonly located at the thoracolumbar junction. Vertebral fractures were attributed to osteoporosis (approximately 40% of participants had a spine *T*-score of <−2.5).[10,20] At baseline, participants reported severe back pain (mean visual analogue scale [VAS][22] score 6.8), substantial back-related disability (mean Rolland-Morris Disability Questionnaire [RMDQ][23] score 17.5), and poor health-related quality of life (mean Short Form-36 Physical Component Summary [SF-36 PCS][24] score 25.7 and mean EuroQol [EQ-5D][25] score 0.18). In Yi and colleagues,[21] mean age was 61.3 years and 62% were female. Qualifying vertebral fractures were described as symptomatic. However, no data were reported on the age, location or number of vertebral fractures per participant, prevalence of osteoporosis, or participant baseline pain, disability, or quality of life.

Outcome measures

Back pain

BK was associated with significantly more reduction in pain than NSM at all time points, though the relative difference between groups in improvement in VAS appeared to diminish over time: MD in decline from baseline at 30 days (−1.82; 95% CI, −2.37 to −1.27; $n = 264$ participants); at 3 months (−1.45; 95% CI, −2.01 to −0.89; $n = 246$); at 6 months (−1.48; 95% CI, −2.05 to −0.91; $n = 241$); at 12 months (−0.84; 95% CI, −1.42 to −0.26; $n = 226$); and at 24 months (−0.69; 95% CI, −1.27 to −0.11; $n = 200$) (Supporting Table 1).

Back-related disability

BK was associated with significantly more reduction in RMDQ than NSM at 30 days (−4.20; 95% CI, −5.54 to −2.86; $n = 255$), 3 months (−3.69; 95% CI, −5.10 to −2.28; $n = 225$), 6 months (−3.05; 95% CI, −4.50 to −1.60; $n = 230$), and 12 months (−2.90; 95% CI, −4.37 to −1.43; $n = 204$), but not at 24 months (−1.43; 95% CI, −2.91 to 0.05; $n = 193$). The relative reduction in disability after BK compared with NSM appeared to diminish with time (Supporting Table 2).

Quality of life

BK was associated with significantly more improvement than NSM on SF-36 PCS at 30 days (5.40; 95% CI, 3.14 to 7.66; $n = 261$), 3 months (4.00; 95% CI, 1.67 to 6.33; $n = 241$), and 6 months (3.30; 95% CI, 1.00 to 5.60; $n = 237$), but not at 12 months (1.60; 95% CI, −0.73 to 3.93; $n = 225$) or 24 months (1.50; 95% CI, −0.83 to 3.83; $n = 186$) (Supporting Table 3). By comparison, BK was associated with significantly more improvement than NSM on

Table 1. Study Characteristics

Study	Blinding	Follow-up (months)	Centers	Countries	Participants	Randomisation	Outcome measures[a]	Funding
BK versus NSM								
Wardlaw (2009); Boonen (2011); Borgstrom (2013); Van Meirhaeghe (2013) (FREE Trial)	Unblinded	24	Multiple	Belgium, Germany, Sweden, Scotland	300	Computer-generated permuted block	SF-36 PCS,[a] EQ5D, VAS, analgesic use RMDQ, patient satisfaction, TUG test, new VCF, kyphosis angle, RAD, QALY, vertebral height, AE	Medtronic Spine, LLC
Yi (2014)	Single blind (outcome assessor)	48	Single	China	200	Both stated "randomized" and "surgeon...blindly chose...treatment"	New VCF,[a] AE	No disclosures
BK versus PV								
Du (2006)	Not specified	24	Single	China	86	Quasi-randomized (divided according to the surgeon)	VAS, ODI, vertebral height, participant satisfaction, kyphotic angle, new VCF	No disclosures
Liu (2010, 2015)	Single blind (radiology techs)	60	Single	Taiwan	100	Permuted block	Vertebral height, kyphotic angle, VAS, new VCF	University/hospital grant
Vogl (2013)	Single blind (participants)	12	Multiple	Germany, USA	77	Stated only as "randomized"	Cement leakage,[a] vertebral height, new VCF	Soteira, Inc.
Dohm (2014) (KAVIAR Trial)	Single blind (radiology techs)	24	Single	USA	404	Computer generated dynamic minimisation	New VCF,[a] SF-36 PCS, EQ-5D, ODI, VAS, kyphosis correction, AE	Medtronic Spine, LLC
Wang (2015)	Blinded (participants, radiologists)	12	Single	China	107	Stated only as "randomized"	VAS, ODI, cement leakage, vertebral height restoration rate, new VCF, AE	No disclosures
BK versus VBS								
Werner (2013)	Unblinded	Postoperation	Single	Switzerland	100	Computer generated block	Kyphotic angle,[a] cement leakage, material-related complications	No disclosures
BK versus KIVA								
Korovessis (2013)	Blinded (participants, investigators, outcome assessors)	14	Single	Greece	163	Stated only as "randomized"	Cement leakage, vertebral height, kyphotic angle, VAS, SF-36, ODI	No disclosures
Tutton (2015) (KAST Trial)	Single blind (participants only until after procedure)	12	Multiple	USA, EU	300	Computer generated block	Composite (reduced VAS by 15 mm on 100 mm VAS, improved or ≤10 point worsening ODI, absence of device-related SAE),[a] VAS, ODI, new VCF	Benvenue Medical, Inc.

BK = balloon kyphoplasty; NSM = nonsurgical management; SF-36 PCS = Short Form 36 Physical Component Summary score; EQ5D = EuroQol 5 dimensions; VAS = visual analogue scale; RMDQ = Rolland Morris Disability questionnaire; TUG = timed up and go; VCF = vertebral compression fracture; RAD = restricted activity days; QALY = quality adjusted life year; AE = adverse event; PV = percutaneous vertebroplasty; ODI = Oswestry disability index; VBS = vertebral body stenting; SAE = serious adverse event.

[a]Specified as primary outcome measure.

Table 2. Study Characteristics of Included Literature

Report (year)	Trial ID	Blinding	Follow-up (months)	Centers	Countries	n	Randomization	Intervention	Approach	Comparator	Primary outcome	Secondary outcomes	Funding
Yi (2014)	–	Single blind	48	Single	China	200	Surgeon decision	BK	Bipedicular	NSM	New VCF	AE	No disclosures
Werner (2013)	–	Unblinded	Postoperation	Single	Switzerland	100	5 × Block of 20	BK	Bipedicular	VBS	Postoperative change Cobb's angle	Complications	No disclosures
Wardlaw (2009)	FREE (NCT00211211)	Unblinded	12	Multiple	Belgium, Germany, Sweden, Scotland	300	Permuted block 1:1	BK	Bipedicular	NSM	Difference in change in 1 month SF-36 PCS	EQ5D, VAS, analgesic use RMQD, patient satisfaction, TUG test, fracture, Cobb's angle, RAD, vertebral body height, AE	Medtronic Spine, LLC
Wang (2015)	–	Single blind	12	Single	China	107	n/sp	HVCV	Unipedicular	BK	VAS, ODI	Compression ratio, leakage	No disclosures
Vogl (2013)	–	Unblinded	12	Multiple	Germany, USA	77	n/sp	BK	Bipedicular	PV	Cement leakage	Vertebral height, new fractures, other complications	Soteira, Inc. (Natick, MA)
Van Meirhaeghe (2013)	FREE (NCT00211211)	Unblinded	24	Multiple	Belgium, Germany, Sweden, Scotland	300	Permuted block 1:1	BK	Bipedicular	NSM	1-month SF-36 PCS	EQ5D, VAS, RMQD, patient satisfaction, TUG test, fracture, Cobb's angle, vertebral body height, AE	Medtronic Spine, LLC
Tutton (2015)	KAST (NCT01123512)	Single blind	12	Multiple	USA (n = 15); EU (n = 6)	253/300	Stratified by site, number of intended treatment levels level; Block allocation	KIVA	Bipedicular	BK	Composite (reduce VAS by 15mm on 100 mm VAS; maintain/improve ODI; absence of SAE)	Volume bone cement used, VAS score, ODI, adjacent level fracture rates	Benvenue Medical, Inc.
Liu (2015)	–	n/a	60	Single	Taiwan	100	"Divided equally"	BK	Bipedicular	PV	Vertebral body height, Cobb's angle, and VAS	n/r	University/hospital grant
Liu (2010)	–	n/a	6	Single	Taiwan	100	Permuted block 1:1	BK	Bipedicular	PV	Vertebral body height, Cobb's angle, and VAS	n/r	University/hospital grant
Korovessis (2013)	–	Single blind	14	Single	Greece	163	Block	KIVA	Bipedicular	BK	AVBHr, MVBHr, PVBHr	VAS, SF36, ODI	No disclosures
Du (2014)	–	n/a	24	Single	China	86	Surgeon decision	BK	Bipedicular	PV	VAS, ODI	Vertebral height, patient satisfaction, Cobb's angle, new VCF	No disclosures
Dohm (2014)	KAVIA R (NCT00323609)	Unblinded	24	Single	USA	404	Computer-generated dynamic minimization	BK	Bipedicular	PV	New radiographic VCF	SF-36, EQ5D, RMQD, ODI, VAS	Medtronic Spine, LLC
Borgstrom (2013)	FREE (NCT00211211)	Unblinded	24	Multiple	Belgium, Germany, Sweden, Scotland	300	Permuted block 1:1	BK	Bipedicular	NSM	Difference in change in 1 month SF-36 PCS	EQ5D, VAS, analgesic use RMQD, patient satisfaction, TUG test, fracture, Cobb's angle, RAD, vertebral body height, AE, QALY	Medtronic Spine, LLC
Boonen (2011)	FREE (NCT00211211)	Unblinded	24	Multiple	Belgium, Germany, Sweden, Scotland	300	Permuted block 1:1	BK	Bipedicular	NSM	Difference in change in 1 month SF-36 PCS	EQ5D, VAS, analgesic use RMQD, patient satisfaction, TUG test, fracture, Cobb's angle, RAD, vertebral body height, AE	Medtronic Spine, LLC

BK = balloon kyphoplasty; NSM = nonsurgical management; VCF = vertebral compression fracture; AE = adverse event; VBS = vertebral body stenting; SF-36 PCS = Short Form 36 Physical Component Summary score; EQ5D = EuroQol 5 dimensions; VAS = visual analogue scale; RMQD = Rolland Morris Disability questionnaire; TUG = timed up and go; RAD; restricted activity days; n/sp = not specified; HVCV = high viscosity cement vertebroplasty; ODI = Oswestry disability index; PV = percutaneous kyphoplasty; EU = European Union; n/r = not reported; SAE = serious adverse event; n/a = not applicable; AVBHr = anterior vertebral body height ratio; MVBHr = midline vertebral body height ratio segmental kyphotic angle (Gardner angle); PVBHr = posterior vertebral body height ratio; QALY = quality adjusted life year.

the EQ-5D at all time points up to 24 months. For both these outcomes, the difference between groups appeared to diminish over time (Supporting Table 4).

Incident vertebral fractures

There was no statistically significant difference between BK and NSM participants in risk of new-onset radiographic vertebral fractures occurring at one month (7.4% versus 4.6%; risk ratio [RR] = 1.59; 95% CI, 0.63 to 4.00; events = 18; n = 300), 3 months (21.9% versus 27.0%; RR = 0.81; 95% CI, 0.51 to 1.29; events = 54; n = 223), 12 months (33.0% versus 25.3%; RR = 1.31; 95% CI, 0.85 to 2.02; events = 62; n = 220), or 24 months (47.5% versus 44.1%; RR = 1.08; 95% CI, 0.81 to 1.44; events = 101; n = 220) (Supporting Table 5); or at 24 months in incident adjacent radiographic vertebral fractures (23.7% versus 16.7%; RR = 1.54; 95% CI, 0.89 to 2.65; events = 45, n = 220) or incident clinical vertebral fractures (20.8% versus 17.9%; RR = 1.07; 95% CI, 0.69 to 1.68; events = 58; n = 300)[10,17,20,21] (Supporting Table 6).

Adverse events

The FREE study reported a significantly increased risk of any AE occurring within 1 month following BK compared with NSM (63.1% versus 36.4%; RR = 1.73; 95% CI, 1.36 to 2.21; events = 149; n = 300), with the most common AEs being back pain, new vertebral fracture, nausea or vomiting, and urinary tract infection.[17] By comparison, there was no difference in risk of any AE within 24 months (89.9% versus 88.7%; RR = 1.01; 95% CI, 0.94 to 1.10; events = 268; n = 300).[10,17,20] There was no significant difference in risk of serious AE, either within 1 month (16.1% versus 11.3%; RR = 1.43; 95% CI, 0.80 to 2.55; events = 41; n = 300) or 24 months of the intervention (49.7% versus 48.3%; RR = 1.03; 95% CI, 0.82 to 1.29; events = 147; n = 300)[10,17,20] (Supporting Table 7).

Kyphoplasty versus vertebroplasty

Study characteristics

Six reports[12,14,15,18,19,21] met eligibility criteria, including five unique RCTs (n = 857) and one quasi-randomized study (n = 112).[13] All six trials were rated as having high risk of bias. Two trials were single-blinded,[19,21] two trials were unblinded,[12,18] and two trials had no blinding specified.[13–15] Treatment allocation was performed by computerized block randomization in two studies,[12,14,15] assigned by the operating surgeon in two studies,[13,21] and was not specified in two studies.[18,19] Follow-up duration ranged from 6 to 60 months[14,15] (Table 2).

Patient characteristics

Mean participant age was 71.6 years and about 75% were female. Qualifying VCF were acute (<6 weeks old) or subacute (6 to 12 weeks old),[12–15] with some studies requiring or reporting supportive findings on MRI.[12,13,19,21] Fractures were most commonly located near the thoracolumbar junction. Three studies limited participation to individuals who had failed several weeks of conservative therapy.[13,18,19] Three studies were limited to or were mostly composed of participants with osteopenia or osteoporosis.[12,18,19] At baseline, participants reported severe back pain (mean VAS range, 7.6 to 8.1),[12–15,19] substantial back-related disability (mean Oswestry Disability Index [ODI][26] range, 58% to 66%),[12,13,19] and fair to poor quality of life (mean SF-36 PCS and EQ-5D approximately 28 and 0.42, respectively).[12]

Outcome measures

Back pain

In two RCTs, results favored BK over vertebroplasty (PV) at 30 days VAS MD = −0.28; 95% CI, −0.43 to −0.13; n = 107, k = 1 trial)[19] and favored PV over BK at 5 years (0.60; 95% CI, 0.09 to 1.11; n = 100, k = 1 trial),[14] but there were no statistically significant differences at other time points[12,15,19] (Fig. 2). In the quasi-randomized study, there were no statistically significant differences (p < 0.05) in VAS scores between treatment groups at any follow-up time point, but there was a statistically significant difference between groups in change from baseline to 2-year follow-up (0.60; 95% CI, 0.22 to 0.98; n = 86) that was small and not likely to have been clinically meaningful.[13]

Back-related disability

In two RCTs[12,19] and one quasi-randomized study,[13] there was no statistically significant difference between treatments in improvement in ODI from baseline to any time points ranging between 3 months and 2 years (Supporting Table 8).

Quality of life

There was no statistically significant difference between treatments in improvement in SF-36 PCS or EQ-5D at any time point[12] (Supporting Tables 9 and 10).

Incident vertebral fractures

There was no statistically significant difference between BK and PV in risk of incident radiographic vertebral fractures occurring within 3 months of intervention (23.3% versus 27.4%; RR = 0.85; 95% CI, 0.58 to 1.26; k = 1),[12] 12 months (28.3% versus 31.5%; RR = 0.89; 95% CI, 0.66 to 1.19; k = 2),[12,19] or 24 months (49.1% versus 57.7%; RR = 0.85; 95% CI, 0.66 to 1.09; k = 1) (Fig. 3).[12] Similarly, there was no significant difference in risk of incident adjacent radiographic vertebral fracture occurring up to 12 months (6.0% versus 7.0%; RR = 0.91; 95% CI, 0.39 to 2.15; n = 278; k = 3),[14,15,18,19] 24 months (16.0% versus 14.0%; RR = 1.14; 95% CI, 0.45 to 2.91; n = 100; k = 1),[14] or 60 months (16.0% versus 14.0%; RR = 1.14; 95% CI, 0.45 to 2.91; n = 100; k = 1) (Fig. 4).[14] There was no significant difference in risk of incident clinical vertebral fracture at 1 month (4.7% versus 8.9%; RR = 0.53; 95% CI, 0.24 to 1.15; k = 1),[12] or 12 months (16.3% versus 22.9%; RR = 0.77; 95% CI, 0.53 to 1.11; k = 2)[12,18] (Supporting Table 11), or, in one quasi-randomized study, at 2 years (18.2% versus 14.3%; RR = 1.27; 95% CI, 0.48 to 3.36; n = 86).[13]

Adverse events

Only one study reported data on adverse events, and found no increased risk of serious AE at 30 days (26.2% versus 27.4%, p = 0.82).[12,27] This trial reported the most common individual types of AEs within 30 days of surgery as procedural pain 6% for BK versus 5% for PV, back pain 7% for BK versus 15% for PV, and
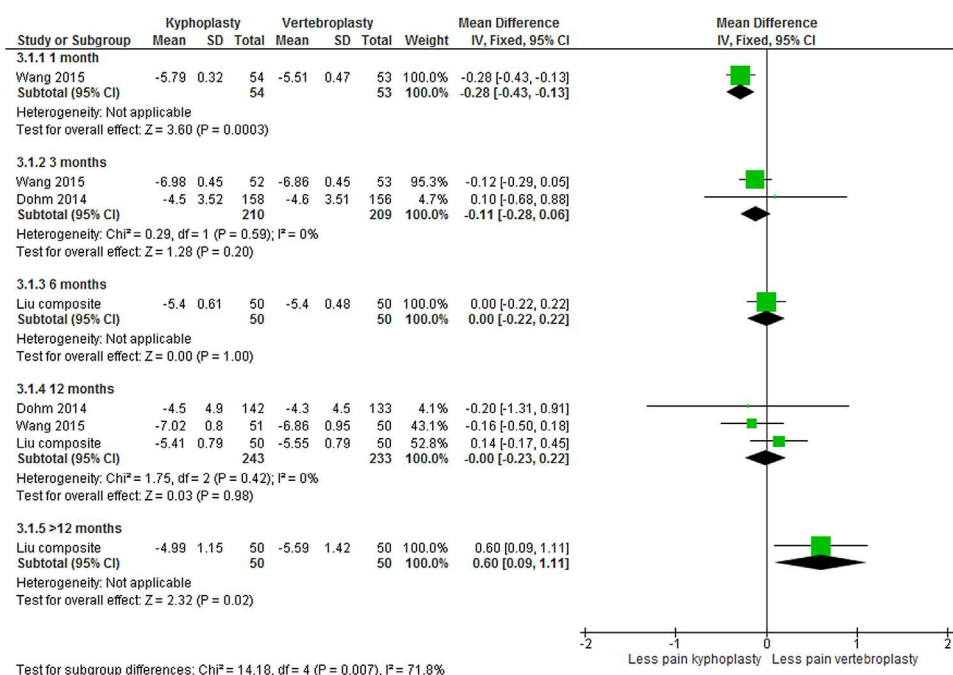
Fig. 2. Mean changes in back pain scores from baseline at 1, 3, 6, 12, and >12 months in kyphoplasty versus percutaneous vertebroplasty study groups.

new symptomatic fracture 5% for BK versus 9% for PV. Within 2 years of surgery, there did not appear to be a significant difference between treatment groups in risk of falls, pneumonia, bronchitis, or urinary tract infection.

Kyphoplasty versus VBS

One eligible trial ($n = 63$, 100 treated levels) randomized participants to BK versus VBS, and reported only that there were no neurologic sequelae in the immediate postoperative period[4] (Table 2). This trial was rated as having high risk of bias for lack of blinding of outcome assessment.

Kyphoplasty versus KIVA®

Study characteristics

Two eligible trials randomized participants to BK versus KIVA®.[5,16] One trial was blinded to participants, investigators, and outcome assessors and was rated as having unclear risk of bias due to unclear allocation of treatment assignment,[5] whereas the other was blinded only to participants, and then only until after the procedure was completed,[16] and was therefore rated as having high risk of bias. Follow-up was 14 and 12 months, respectively (Table 2).

Patient characteristics

Mean participant age was 73.7 years and 72.8% were female. Qualifying vertebral fractures were acute or subacute, and, in the one study that reported location, most commonly around the thoracolumbar junction.[16] One trial limited participation to individuals who had failed conservative treatment, and reported a mean spine T-score in the osteopenic range. At baseline, participants reported severe back pain (mean VAS 8.6) and considerable back-related disability (mean ODI 63%).[16]

Outcomes

Back pain, back-related disability, and quality of life

There was no difference between treatment groups in the proportion of participants with >5.5 points improvement in back pain (VAS) (43% for BK versus 54% for KIVA®; RR = 0.80; 95% CI, 0.58 to 1.10),[5] with ≥1.5 points improvement in back pain (VAS) (97.6% versus 95.3%),[16] or with undefined "improved" SF-36 PCS (59% for BK versus 51% for KIVA®).[5] There also was no between-group difference in achievement of a composite endpoint requiring a 1.5 point improvement in VAS, and absence of either a >10 point worsening in ODI or a device-related AE,[16] and no between-group difference in mean improvement from baseline in back pain (VAS),[5,16] back-related disability (ODI),[5,16] or quality of life (SF-36 PCS).[5,16]

Incident vertebral fractures

There was no difference between BK and KIVA® participants in risk of incident radiographic vertebral fractures (12.8% versus 12.2%, $p = 0.91$) or incident adjacent radiographic vertebral fractures (17.1% versus 15.7%, $p = 0.64$).[5]

Adverse events

There was no difference in serious adverse events between BK and KIVA® participants through 12 months (34.6% versus 28.6%; RR = 1.21; 95% CI, 0.84 to 1.75; events = 80; $n = 253$).[16] Individual types of AEs were reported only if judged by trial investigators to be procedure-related, including three participants randomized to KIVA® with herpes zoster, postprocedural pain, and pruritus, respectively, and four assigned to BK with an airway complication, back pain, ischemic stroke, and rash, respectively.[16]
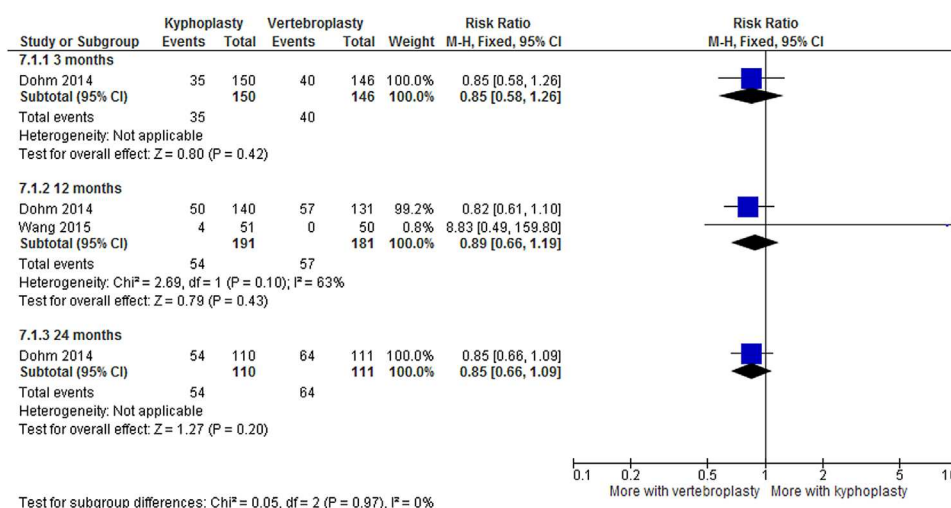
Fig. 3. Incident radiographic vertebral fractures at 3, 12, and >12 months (number of events through to time point) in kyphoplasty versus percutaneous vertebroplasty study groups.
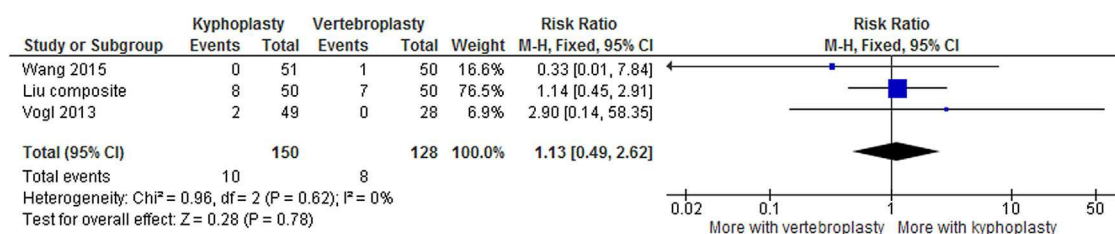
Fig. 4. Incident adjacent radiographic vertebral fractures at >12 months (number of events through to time point) in kyphoplasty versus percutaneous vertebroplasty study groups.

Discussion

On average, individuals with painful VCF experienced statistically significant symptomatic improvement compared with baseline with all studied treatment interventions, including NSM. Though we found that BK was associated with improved pain, back-related disability, and quality of life outcomes compared with NSM, these results were derived almost entirely from a single trial. Further, magnitude of improvement from baseline in these outcome measures after BK relative to NSM appeared to diminish over time, mean between group differences were smaller than previously reported minimally clinically important differences in a population of individuals being treated for chronic low back pain,[28] raising concerns about their clinical significance. Because we identified no eligible trials of BK versus sham BK, it also was not possible to determine to what extent the observed improvements of BK versus NSM were attributable to a placebo effect.

Compared with NSM, BK was not associated with a statistically significantly increased risk of incident VCF, though CIs were wide and results could not exclude a clinically meaningful increase in risk. Further, compared with NSM, BK was associated with a near doubling in risk of any AE within 30 days of intervention. Based on the mean between-group differences in efficacy outcomes of

uncertain clinical importance, an increase in early AE, and the high risk of bias of the largest eligible BK versus NSM trial, it is uncertain whether any benefits of BK versus NSM for treatment of VCF outweigh potential harms, both in the VCF population overall and within selected patient subgroups.

We found no significant difference between BK and either PV or KIVA® in pain, back-related disability, or quality of life outcomes, or in risk of incident VCF, or risk of any AE or serious AE. These results were limited by the lack of results reporting the proportion of participants in each treatment group that experienced a clinically important difference in each efficacy outcome, wide CIs around the estimates for risk of incident VCF that could not exclude clinically important differences in fracture risk, and limited reporting of AE outcomes. The high risk of bias ratings of all the trials that compared BK versus PV further limits confidence in these findings.

Uncertainty about the benefits and harms of BK relative to NSM, sham BK, PV, or other treatments is further complicated by uncertainty about the effect of PV. A prior systematic review found no statistically significant difference between PV and sham PV in mean change from baseline to 1 month in either pain or back-related disability,[29] both overall and in two participant subgroups postulated to be more likely to benefit from PV—those with recent VCF (<6 weeks) or with severe baseline back

pain (score on 0 to 10 scale ≥8). Further analysis suggested that compared with participants randomized to sham PV, those assigned to PV may have been slightly more likely to experience clinically meaningful improvements in pain at 1 month (reduction in pain score of ≥3: RR = 1.3 [95% CI, 0.8 to 1.9]; reduction in pain score of ≥30%: RR = 1.3 [95% CI, 1.0 to 1.8]). A more recent RCT of PV versus sham PV for treatment of acute, severely painful VCF reported that participants assigned PV were significantly more likely than those in the sham PV group to have a clinically meaningful reduction in pain score between baseline and follow-up time points through 6 months.[30] It is uncertain whether the apparent differences in outcomes between the earlier and more recent PV versus sham PV trials are attributable to methodological differences, including the lack of numbing of the periosteum and the greater volume of bone cement used in the recent trial. Combined with the differences in participant characteristics and in the PV intervention groups between the recent PV versus sham PV trial and those in prior BK versus PV trials, it seems unwise to make indirect comparisons between these sets of studies to draw inferences about the unstudied comparison of BK versus sham BK.

The current review was limited by available evidence. Though 10 unique trials met eligibility criteria, after considering the different BK treatment comparisons, outcome measures and time points, only relatively few participants provided information about the efficacy and safety of BK versus other interventions. Second, because all but two trials reported results for efficacy outcomes only as overall group means,[5,16] it was difficult to determine how many and which types of participants achieved clinically meaningful improvements with treatment. Third, AEs and incident vertebral fractures were rarely systematically reported and often were not reported at all, particularly for individual types of AEs. This hampered our ability to weigh the relative harms of BK against any potential benefits. Fourth, most trials were rated as having high risk of bias, most commonly due to lack of blinding of participants and/or outcome assessors, and less often due to a lack of allocation concealment, both which could have led to overestimation of the true effect of interventions.

In conclusion, we found that in middle-aged and older adults with VCF, based on only a single RCT, BK was associated with greater improvement in pain, disability, and quality of life, and an increase in risk of early AE compared with NSM. However, the magnitude of treatment differences may have been too small to be clinically meaningful, diminished over time, and likely was attributable at least in part to a placebo effect. Based on a small number of heterogeneous (and high risk of bias) studies, there was no difference in these outcomes between BK and either the PV or KIVA vertebral augmentation techniques. Risks of subsequent fracture were not statistically significantly different between BK and other treatments, but results could not rule out important differences. These remaining areas of uncertainty should be addressed by future trials that randomize participants with acute and subacute painful VCF to BK versus sham BK, PV, or KIVA, mask study participants, investigators and outcome assessors, follow participants for at least 1 year, include adequately powered responder analyses for efficacy outcomes (eg, proportion achieving a clinically important improvement in back pain), systematically collect results for clinical and radiographic vertebral fracture and AE, and include a priori subgroup analyses for patient groups with characteristics suspected to modify the effects of treatment.

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Supplementary table 1. Outcome data for VAS back pain in the comparison BK vs. NSM

Time   1 Month

Study	BK Mean VAS change from baseline	SD	n	NSM Mean VAS change from baseline	SD	n	Weight	Effect [95%CI]
FREE	-3.27	2.27	136	-1.45	2.28	128	100.00%	-1.82 [-2.37, -1.27]
Subtotal (95% CI)			136			128	100.00%	-1.82 [-2.37, -1.27]

Heterogeneity: Not applicable. Test for overall effect: Z = 6.50 (P < 0.00001)

3 months

Study								
FREE	-3.86	2.25	132	-2.41	2.23	114	100.00%	-1.45 [-2.01, -0.89]
Subtotal (95% CI)			132			114	100.00%	-1.45 [-2.01, -0.89]

Heterogeneity: Not applicable. Test for overall effect: Z = 5.06 (P < 0.00001)

6 months

Study								
FREE	-4.06	2.25	128	-2.58	2.25	113	100.00%	-1.48 [-2.05, -0.91]
Subtotal (95% CI)			128			113	100.00%	-1.48 [-2.05, -0.91]

Heterogeneity: Not applicable. Test for overall effect: Z = 5.10 (P < 0.00001)

12 months

Study								
FREE	-3.98	2.27	121	-3.14	2.19	105	100.00%	-0.84 [-1.42, -0.26]
Subtotal (95% CI)			121			105	100.00%	-0.84 [-1.42, -0.26]

Heterogeneity: Not applicable. Test for overall effect: Z = 2.83 (P = 0.005)

>12 months

Study								
FREE	-3.97	2.17	111	-3.28	2.02	89	100.00%	-0.69 [-1.27, -0.11]
Subtotal (95% CI)			111			89	100.00%	-0.69 [-1.27, -0.11]

Heterogeneity: Not applicable. Test for overall effect: Z = 2.32 (P = 0.02)

Supplementary table 2. Outcome data for RMDQ back disability in the comparison BK vs. NSM

Time   1 Month

	BK Mean RMQD change from baseline	SD	n	NSM Mean RMQD change from baseline	SD	n	Weight	Effect [95%CI]
Study	baseline	SD	n	baseline	SD	n	Weight	Effect [95%CI]
FREE	10.9	5.5	129	15.1	5.39	124	100.00%	-4.20 [-5.54, -2.86]
Subtotal (95% CI)			129			124	100.00%	-4.20 [-5.54, -2.86]

Heterogeneity: Not applicable. Test for overall effect: Z = 6.13 (P < 0.00001)

3 months

FREE	9.21	5.48	118	12.9	5.27	107	100.00%	-3.69 [-5.10, -2.28]
Subtotal (95% CI)			118			107	100.00%	-3.69 [-5.10, -2.28]

Heterogeneity: Not applicable. Test for overall effect: Z = 5.15 (P < 0.00001)

6 months

FREE	8.45	5.4	111	11.5	5.59	109	100.00%	-3.05 [-4.50, -1.60]
Subtotal (95% CI)			111			109	100.00%	-3.05 [-4.50, -1.60]

Heterogeneity: Not applicable. Test for overall effect: Z = 4.11 (P < 0.0001)

12 months

FREE	8.6	5.33	103	11.5	5.39	101	100.00%	-2.90 [-4.37, -1.43]
Subtotal (95% CI)			103			101	100.00%	-2.90 [-4.37, -1.43]

Heterogeneity: Not applicable. Test for overall effect: Z = 3.86 (P = 0.0001)

>12 months

FREE	8.87	5.41	103	10.3	5.08	90	100.00%	-1.43 [-2.91, 0.05]
Subtotal (95% CI)			103			90	100.00%	-1.43 [-2.91, 0.05]

Heterogeneity: Not applicable. Test for overall effect: Z = 1.89 (P = 0.06)

Supplementary table 3. Outcome data for SF-36 (PCS) quality of life in the comparison BK vs. NSM

Time   1 Month

Study	BK Mean SF-36 change from baseline	SD	n	NSM Mean SF-36 change from baseline	SD	n	Weight	Effect [95%CI]
FREE	7.4	9.52	136	2	9.12	125	100.00%	5.40 [3.14, 7.66]
Subtotal (95% CI)			136			125	100.00%	5.40 [3.14, 7.66]

Heterogeneity: Not applicable. Test for overall effect: Z = 4.68 (P < 0.00001)

3 months

Study		SD	n		SD	n	Weight	Effect [95%CI]
FREE	9.6	9.34	131	5.6	9.09	110	100.00%	4.00 [1.67, 6.33]
Subtotal (95% CI)			131			110	100.00%	4.00 [1.67, 6.33]

Heterogeneity: Not applicable. Test for overall effect: Z = 3.36 (P = 0.0008)

6 months

Study		SD	n		SD	n	Weight	Effect [95%CI]
FREE	10.4	9.19	127	7.1	8.82	110	100.00%	3.30 [1.00, 5.60]
Subtotal (95% CI)			127			110	100.00%	3.30 [1.00, 5.60]

Heterogeneity: Not applicable. Test for overall effect: Z = 2.82 (P = 0.005)

4.2.4 12 months

12 months

Study		SD	n		SD	n	Weight	Effect [95%CI]
FREE	9.9	8.9	119	8.3	8.92	106	100.00%	1.60 [-0.73, 3.93]
Subtotal (95% CI)			119			106	100.00%	1.60 [-0.73, 3.93]

Heterogeneity: Not applicable. Test for overall effect: Z = 1.34 (P = 0.18)

>12 months

Study		SD	n		SD	n	Weight	Effect [95%CI]
FREE	9.8	8.32	104	8.3	7.85	82	100.00%	1.50 [-0.83, 3.83]
Subtotal (95% CI)			104			82	100.00%	1.50 [-0.83, 3.83]

Heterogeneity: Not applicable. Test for overall effect: Z = 1.26 (P = 0.21)

Supplementary table 4. Outcome data for EQ5D quality of life in the comparison BK vs. NSM

Time 1 Month

Study	BK Mean EQ5D change from baseline	SD	n	NSM Mean EQ5D change from baseline	SD	n	Weight	Effect [95%CI]
FREE	0.54	33	144	0.37	0.34	149	100.00%	0.17 [-5.22, 5.56]
Subtotal (95% CI)			144			149	100.00%	0.17 [-5.22, 5.56]

Heterogeneity: Not applicable. Test for overall effect: Z = 0.06 (P = 0.95)

3 months

FREE	0.59	35	136	0.49	0.31	125	100.00%	0.10 [-5.78, 5.98]
Subtotal (95% CI)			136			125	100.00%	0.10 [-5.78, 5.98]

Heterogeneity: Not applicable. Test for overall effect: Z = 0.03 (P = 0.97)

6 months

FREE	0.63	0.32	131	0.5	0.29	111	100.00%	0.13 [0.05, 0.21]
Subtotal (95% CI)			131			111	100.00%	0.13 [0.05, 0.21]

Heterogeneity: Not applicable. Test for overall effect: Z = 3.31 (P = 0.0009)

12 months

FREE	0.61	0.3	120	0.51	0.31	106	100.00%	0.10 [0.02, 0.18]
Subtotal (95% CI)			120			106	100.00%	0.10 [0.02, 0.18]

Heterogeneity: Not applicable. Test for overall effect: Z = 2.46 (P = 0.01)

>12 months

FREE	0.61	0.29	111	0.53	0.29	91	100.00%	0.08 [-0.00, 0.16]
Subtotal (95% CI)			111			91	100.00%	0.08 [-0.00, 0.16]

Heterogeneity: Not applicable. Test for overall effect: Z = 1.95 (P = 0.05)

Supplementary table 5. Outcome data for incident radiographic fracture in the comparison BK vs. NSM Radiographic

30 days

	Number of events through to timepoint	n	Number of events through to timepoint	n	Weight	Effect [95% CI]
FREE	11	138	7	128	100.0%	1.46 [0.58, 3.65]
Subtotal (95% CI)		138		128	100.0%	1.46 [0.58, 3.65]
Total events	11		7			

Heterogeneity: Not applicable. Test for overall effect: Z = 0.81 (P = 0.42)

3 months

FREE	27	123	27	100	100.0%	0.81 [0.51, 1.29]
Subtotal (95% CI)		123		100	100.0%	0.81 [0.51, 1.29]
Total events	27		27			

Heterogeneity: Not applicable. Test for overall effect: Z = 0.88 (P = 0.38)

12 months

FREE	38	115	24	95	100.0%	1.31 [0.85, 2.02]
Subtotal (95% CI)		115		95	100.0%	1.31 [0.85, 2.02]
Total events	38		24			

Heterogeneity: Not applicable. Test for overall effect: Z = 1.22 (P = 0.22)

24 months

FREE	56	118	45	102	100.0%	1.08 [0.81, 1.44]
Subtotal (95% CI)		118		102	100.0%	1.08 [0.81, 1.44]
Total events	56		45			

Heterogeneity: Not applicable. Test for overall effect: Z = 0.49 (P = 0.62)

Supplementary table 6. Outcome data for incident fractures in the comparison BK vs. NSM

Clinical

Late	Number of events through to timepoint	n	Number of events through to timepoint	n	Weight	Effect [95%CI]
FREE	31	120	27	112	67.5%	1.07 [0.69, 1.68]
Yi 2014	5	79	17	121	32.5%	0.45 [0.17, 1.17]
Total (95% CI)		199		233	100.0%	0.87 [0.58, 1.30]
Total events	36		44			

Heterogeneity: Chi² = 2.66, df = 1 (P = 0.10); I² = 62%. Test for overall effect: Z = 0.68 (P = 0.50)

Adjacent

Late						
FREE	28	120	17	112	100.0%	1.54 [0.89, 2.65]
Total (95% CI)		120		112	100.0%	1.54 [0.89, 2.65]
Total events	28		17			

Heterogeneity: Not applicable. Test for overall effect: Z = 1.55 (P = 0.12)

Supplementary table 7. Outcome data for adverse events in the comparison BK vs. NSM

Early	BK			NSM			
Study	Number of events through to timepoint	n		Number of events through to timepoint	n	Weight	Effect [95% CI]
FREE	94	149		55	151	100.0%	1.73 [1.36, 2.21]
Total (95% CI)		149			151	100.0%	1.73 [1.36, 2.21]
Total events	94			55			

Heterogeneity: Not applicable. Test for overall effect: Z = 4.41 (P < 0.0001)

Late							
FREE	134	149		134	151	100.0%	1.01 [0.94, 1.10]
Total (95% CI)		149			151	100.0%	1.01 [0.94, 1.10]
Total events	134			134			

Heterogeneity: Not applicable. Test for overall effect: Z = 0.33 (P =0.74)

Serious adverse events							
Early							
FREE	24	149		17	151	100.0%	1.43 [0.80, 2.55]
Total (95% CI)		149			151	100.0%	1.43 [0.80, 2.55]
Total events	24			17			

Heterogeneity: Not applicable. Test for overall effect: Z = 1.21 (P =0.23)

Late							
FREE	74	149		73	151	100.0%	1.03 [0.82, 1.29]
Total (95% CI)		149			151	100.0%	1.03 [0.82, 1.29]
Total events	74			73			

Heterogeneity: Not applicable. Test for overall effect: Z = 0.23 (P =0.82)

Supplementary table 8. Outcome data for back disability (ODI) in the comparison BK vs. PV

3 months	BK			PV				
Study	Mean ODI change from baseline	SD	n	Mean ODI change from baseline	SD	n	Weight	Effect [95%CI]
Dohm 2014	-28.4	19.56	153	-25.2	19.68	141	21.6%	-3.20 [-7.69, 1.29]
Wang 2015	-52.12	5.89	52	-51.48	6.44	53	78.4%	-0.64 [-3.00, 1.72]
Subtotal (95% CI)			205			194	100.0%	-1.19 [-3.28, 0.89]

Heterogeneity: Chi² = 0.98, df = 1 (P = 0.32); I² = 0%. Test for overall effect: Z = 1.12 (P = 0.26)

12 months								
Dohm 2014	-28.8	20.3	138	-28.0	19.75	119	32.0%	-0.80 [-5.71, 4.11]
Wang 2015	-55.1	6.7	51	-54.15	6.43	20	68.0%	-0.95 [-4.31, 2.41]
Subtotal (95% CI)			189			139	100.0%	-0.90 [-3.68, 1.87]

Heterogeneity: Chi² = 0.00, df = 1 (P = 0.96); I² = 0%. Test for overall effect: Z = 0.64 (P = 0.52)

>12 months								
Dohm 2014	-26.9	21.4	108	-25.9	21.15	93	100.0%	-1.00 [-6.90, 4.90]
Subtotal (95% CI)			108			93	100.0%	-1.00 [-6.90, 4.90]

Heterogeneity: Not applicable. Test for overall effect: Z = 0.33 (P = 0.74)

Supplementary table 9. Outcome data for SF-36 (PCS) quality of life in the comparison BK vs. PV

3 Months

Study	Mean SF-36 change from baseline	SD	n	Mean SF-36 change from baseline	SD	n	Weight	Effect [95%CI]
Dohm 2014	8.0	10.72	153	8.3	11.08	138	100.0%	-0.30 [-2.81, 2.21]
Subtotal (95% CI)			153			138	100.0%	-0.30 [-2.81, 2.21]

Heterogeneity: Not applicable. Test for overall effect: Z = 0.23 (P = 0.81)

12 months

Study	Mean SF-36 change from baseline	SD	n	Mean SF-36 change from baseline	SD	n	Weight	Effect [95%CI]
Dohm 2014	8.1	10.4	138	9.6	11.08	118	100.0%	-1.50 [-4.15, 1.15]
Subtotal (95% CI)			138			118	100.0%	-1.50 [-4.15, 1.15]

Heterogeneity: Not applicable. Test for overall effect: Z = 1.11 (P = 0.27)

>12 months

Study	Mean SF-36 change from baseline	SD	n	Mean SF-36 change from baseline	SD	n	Weight	Effect [95%CI]
Dohm 2014	7.6	11.66	108	7.5	11.01	92	100.0%	0.10 [-3.05, 3.25]
Subtotal (95% CI)			108			92	100.0%	0.10 [-3.05, 3.25]

Heterogeneity: Not applicable. Test for overall effect: Z = 0.06 (P = 0.95)

Supplementary table 10. Outcome data for EQ-5D quality of life in the comparison BK vs. PV

3 months	Mean EQ5D change from baseline	SD	n	Mean EQ5D change from baseline	SD	n	Weight	Effect [95%CI]
Dohm 2014	0.29	0.25	152	0.32	0.27	140	100.0%	-0.03 [-0.09, 0.03]
Subtotal (95% CI)			152			140	100.0%	-0.03 [-0.09, 0.03]

Heterogeneity: Not applicable. Test for overall effect: Z = 0.98 (P = 0.33)

12 months								
Dohm 2014	0.3	0.29	137	0.32	0.25	119	100.0%	-0.02 [-0.09, 0.05]
Subtotal (95% CI)			137			119	100.0%	-0.02 [-0.09, 0.05]

Heterogeneity: Not applicable. Test for overall effect: Z = 0.59 (P = 0.55)

>12 months								
Dohm 2014	0.28	0.31	108	0.31	0.24	94	100.0%	-0.03 [-0.11, 0.05]
Subtotal (95% CI)			108			94	100.0%	-0.03 [-0.11, 0.05]

Heterogeneity: Not applicable. Test for overall effect: Z = 0.77 (P = 0.44)

Supplementary table 11. Outcome data for incident vertebral fractures in the comparison BK vs. PV							
Type of fracture		Kyphoplasty		Vertebroplasty			
Radiographic	Study	Number of events through to timepoint	n	Number of events through to timepoint	n	Weight	Effect[95%CI]
3 months	Dohm 2014	35	150	40	146	100.00%	0.85 [0.58, 1.26]
	Total (95% CI)		150		146	100.00%	0.85 [0.58, 1.26]
	Total events	35		40			
	Heterogeneity: Not applicable						
	Test for overall effect: Z = 0.80 (P = 0.42)						
12 months	Dohm 2014	50	140	57	131	99.20%	0.82 [0.61, 1.10]
	Wang 2015	4	51	0	50	0.80%	8.83 [0.49, 159.80]
	Total (95% CI)		191		181	100.00%	0.89 [0.66, 1.19]
	Total events	54		57			
	Heterogeneity: Chi² = 2.69, df = 1 (P = 0.10); I² = 63%						
	Test for overall effect: Z = 0.79 (P = 0.43)						
24 months	Dohm 2014	54	110	64	111	100.00%	0.85 [0.66, 1.09]
	Subtotal (95% CI)		110		111	100.00%	0.85 [0.66, 1.09]
	Total events	54		64			
	Heterogeneity: Not applicable						
	Test for overall effect: Z = 1.27 (P = 0.20)						
Adjacent							
6 months	Liu composite	2	50	0	50	100.00%	5.00[0.25, 101.58]
	Subtotal (95% CI)		50		50	100.00%	5.00[0.25, 101.58]
	Total events	2		0			
	Heterogeneity: Not applicable						
	Test for overall effect: Z = 1.05 (P = 0.29)						

12 months	Liu composite	7	50	7	50	71.50%	1.00 [0.38, 2.64]
	Vogl 2013	2	49	1	28	13.00%	1.14 [0.11, 12.05]
	Wang 2015	0	51	1	50	15.50%	0.33 [0.01, 7.84]
	Subtotal (95% CI)		150		128	100.00%	0.91 [0.39, 2.15]
	Total events	9		9			
	Heterogeneity: Chi² = 0.47, df = 2 (P = 0.79); I² = 0%						
	Test for overall effect: Z = 0.20 (P = 0.84)						
24 months	Liu composite	8	50	7	50	100.00%	1.14 [0.45, 2.91]
	Subtotal (95% CI)		50		50	100.00%	1.14 [0.45, 2.91]
	Total events	8		7			
	Heterogeneity: Not applicable						
	Test for overall effect: Z = 0.28 (P = 0.78)						
60 months	Liu composite	8	50	7	50	100.00%	1.14 [0.45, 2.91]
	Subtotal (95% CI)		50		50	100.00%	1.14 [0.45, 2.91]
	Total events	8		7			
	Heterogeneity: Not applicable						
	Test for overall effect: Z = 0.28 (P = 0.78)						
Clinical							
1 month	Dohm 2014	9	191	17	190	100.00%	0.53 [0.24, 1.15]
	Total (95% CI)		191		190	100.00%	0.53 [0.24, 1.15]
	Total events	9		17			
	Heterogeneity: Not applicable						
	Test for overall effect: Z = 1.61 (P = 0.11)						
12 months	Dohm 2014	38	191	50	190	98.80%	0.76 [0.52, 1.10]
	Vogl 2013	1	49	0	28	1.20%	1.74 [0.07, 41.33]

	Total (95% CI)		24 0		21 8	100.00 %	0.77 [0.53, 1.11]
	Total events	39		50			
	Heterogeneity: Chi² = 0.26, df = 1 (P = 0.61); I² = 0%						
	Test for overall effect: Z = 1.40 (P = 0.16)						

Appendix 3:

Rodríguez AJ (2017) "Vascular risk in familial

Mediterranean fever". Anatol J Cardiol. doi:

10.14744/AnatolJCardiol.2016.22571

Vascular risk in familial Mediterranean fever

Familial Mediterranean fever (FMF) is an autosomal recessive disorder resulting in improper leukocyte clearance during inflammation. The disease often presents in early childhood and is characterized by recurrent attacks. Current treatment includes suppression of inflammation by colchicine. Typical to other rheumatological diseases, FMF is characterized by elevated white blood cell (WBC) count, erythrocyte sedimentation rate (ESR), and serum pro-inflammation markers, such as C-reactive protein and fibrinogen (1). These factors are responsible for increased leukocyte trafficking, vascular permeability, and endothelial dysfunction. These cellular consequences can fundamentally alter the elastic properties of blood vessels. Indeed, rheumatological diseases with recurrent inflammatory attacks, such as FMF, are associated with increased arterial stiffness (2).

In this edition of the Anatolian Journal of Cardiology, data of a cohort of young FMF patients is reported in a study titled "Investigation of the arterial stiffness and associated factors in patients with familial Mediterranean fever" by Çakar et al. (3). In this case-controlled study design, 69 FMF patients and 35 controls were retrospectively studied. The former had significantly higher pulse wave velocity (PWV) amongst other arterial stiffness indices., probably because there were 31 smokers (44%) in the FMF group and only 9 smokers (25%) in the control group, though this was not assessed. Interestingly, augmentation index (both brachial and aortic) was significantly lower in the former compared to controls. It is uncertain if this apparent disparate finding is due to the technique used or if it reflects some residual confounding.

Consistent with their hypotheses, leukocytes, WBC, and pro-inflammatory markers were significantly increased in the FMF group, offering an inflammatory mechanism for the findings. Compared to patients with quiescent disease, arterial stiffness indices were increased in those with an active attack. Further, as there was no significant association between genetic variants of FMF and arterial stiffness indices, it was concluded that arterial stiffness reflects the severity of the condition rather than its origin or type. The most significant results were the findings of a strong correlation of PWV with CRP, WBC, ESR, fibrinogen, and neutrophil–leukocyte ratio in individuals with an active attack. There was a significant relationship between PWV and the dise-

ase duration, reflecting disease chronicity. Unfortunately, these results remained unadjusted, and it is therefore not possible to determine which inflammatory measure is the most strongly related to stiffness.

Why are these results significant? As FMF predominantly affects younger people and the disease is persistent and recurrent, adverse changes in the vasculature attributable to the disease may impose a significant increase in lifetime cardiovascular risk (4). Strategies that reduce inflammation in other rheumatological conditions, such as psoriasis, have shown that this can be effective in reducing arterial stiffness (5). No study exists demonstrating this effect in FMF. Overall, this study highlights the paucity of evidence with regards to macrovascular function in this group of patients who may be at increased risk. Given the ease with which the non-invasive technique is employed to measure arterial stiffness, it may be beneficial to consider vascular risk screening as part of a broad primary prevention strategy targeting significant risk factors, including smoking cessation.

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Appendix 4:

Rodríguez AJ, Scott D, Ebeling PR (2017) "Comments

on Li et al.: Meta-analysis of hypertension and

osteoporotic fracture risk in women and men".

Osteoporos Int. doi: 10.1007/s00198-017-4246-2

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LETTER

Comments on Li et al.: Meta-analysis of hypertension and osteoporotic fracture risk in women and men

A. J. Rodríguez[1] · D. Scott[1,2] · P. R. Ebeling[1,2]

Dear Editor,

We read with interest the meta-analysis by Li et al. on the association between hypertension and osteoporotic fractures [1]. There is growing interest in understanding the potential for interaction between bone and the vasculature. In the main analysis, hypertension (heterogeneously reported throughout the included studies and not defined by the authors) was associated with an odds ratio of 1.33 (95% confidence interval 1.25–1.40) for all fractures in men and women. However, several potential issues suggest these results should be interpreted with caution.

Importantly, the authors state that that they "analyzed 10 articles encompassing 28 independent studies, 1,430,431 participants, and 148,048 osteoporotic fracture cases." However, in the main analysis (Figure 2), it appears that effect estimates based on different fracture types from studies reporting data on any fracture, as well as on specific fracture types (e.g., hip or vertebral), were treated as independent studies. That is to say a hip or vertebral fracture would also have been included as an "any fracture" resulting in multiple case reporting for the one event. Indeed, for the study of Yang et al. [2], their methodology states that "All

types of fragility fracture were classified into three main groups: any fracture (at all sites), hip fracture, and clinical vertebral fracture" confirming our concern. It is therefore not appropriate to say that analyses of different types of fractures within the same study are "independent studies." This warrants clarification.

Furthermore, the methods stated that odds ratios from all studies were pooled together. However, some of the included studies were longitudinal in design and thus reported hazard ratios and these hazard ratios were also pooled [3]. Including hazard ratios in the main analysis is not appropriate because they are an examination of time to an event. In short, incidence is not the same as prevalence. Also, both crude and adjusted estimates were included in the main analysis, which again may not be appropriate as crude estimates are subject to confounding. However, some of these limitations have been addressed in sub-group analyses (e.g., stratifying by study type) which overall appear to support the main finding that fractures are highly prevalent in elderly people with hypertension.

Finally, it appears reference 13 is incorrectly cited as the data from Table 1 cannot be found in the source listed in the reference list [4]. The data reported in Table 1 appears to be from a longitudinal study by an author of the same name reporting hazard ratios, rather than odds ratios [2]. The incorrectly cited Yang paper from the list of references (a retrospective case-control study) instead explored whether a history of osteoporotic fractures influenced the risk of hypertension [4], not the reverse hypothesis (that hypertension predicts increased fracture risk) as tested by Li et al.

Overall, the article by Li et al. has highlighted that more research is needed to provide a greater understanding of potential biological mechanisms and in clarifying the bi-directional nature of the relationship between hypertension and osteoporosis.

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Compliance with ethical standards

Conflicts of interest   None.

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Appendix 5:

Rodríguez AJ, Scott D, Ebeling P, Abrahamsen B (2016) "Reply to: systematic review and meta-analysis for the association of bone mineral density and osteoporosis/osteopenia with vascular calcification in women". Int J Rheum Dis. doi: 10.1111/1756-185X.12942

CORRESPONDENCE

Reply to: systematic review and meta-analysis for the association of bone mineral density and osteoporosis/osteopenia with vascular calcification in women

Dear Editor,

We read with interest the study by Zhang and Feng on the association between bone mineral density (BMD) and vascular calcification in post-menopausal women.[1] The results of their meta-analysis of four studies ($n = 1666$ vascular calcification cases and $n = 1190$ controls) demonstrated that both hip and spine BMD were significantly lower in women with vascular calcification. Further, in those with vascular calcification, there was an approximate four and a half-fold increased likelihood of having osteoporosis, and an approximate two-fold increased likelihood of having osteopenia.

The authors conclude that patients with vascular calcification 'have lower lumbar spine and hip BMD and increased risk for developing osteoporosis or osteopenia'. While it is possible that aspects of increased vascular calcification may contribute to declines in bone density (through common risk factors such as age, smoking, low levels of physical activity and chronic inflammation), this conclusion potentially ignores evidence that vascular calcification can also result from dysregulated bone metabolism in ageing.[2–4]

A longitudinal study in postmenopausal women has shown that progression of aortic calcification is greater in those who demonstrate bone loss, compared to those who experience no bone loss.[5] In a Japanese sample of older men and women, ectopic calcium deposition in mitral and aortic valves was related to severe bone loss attributed to osteoporosis.[6] Furthermore, studies conducted in patients with chronic kidney disease (a condition characterized by bone mineral derangement) demonstrate that vascular calcification is a common feature in this population.[7] Additionally, important catabolic bone metabolites such as phosphate (hyperphosphatemia), calcium (hypercalcemia) and fibroblast growth factor 23 are all elevated in chronic kidney disease, further suggestive of a role for bone dysregulation.[8]

Thus, vascular calcification may well be a consequence of age-related declines in bone density, in addition to the possibility that cardiovascular diseases, including vascular calcification, contribute to bone loss. Other consequences of ageing may also explain the lower BMD of those with vascular calcification, particularly given that the included cross-sectional cohort study by El Maghraoui et al.[9] (which contributed over 50% of participants to the meta-analysis), demonstrated that participants with abdominal aortic calcification were approximately 10 years older than those without calcification. A meta-regression accounting for age was not performed in the article by Zhang and Feng and thus one may speculate that the findings may be confounded by the effects of age.

A previous study (not included in the present meta-analysis) of Japanese-American women has conversely reported no association between BMD and aortic calcification,[10] and thus prospective studies investigating associations between bone loss and vascular calcification are needed to clarify the relationship between these conditions. Nevertheless, Zhang and Feng's study highlights the increasing research interest in mechanisms that may lead to both osteoporosis and cardiovascular disease in ageing populations.

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Appendix 6:

Rodriguez AJ, Scott D, Ebeling P (2016) "Effect of weight loss induced by energy restriction on measures of arterial compliance: A systematic review and meta-analysis". Atherosclerosis 252:201–202. doi: 10.1016/j.atherosclerosis.2016.06.043
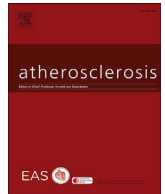
Correspondence

Effect of weight loss induced by energy restriction on measures of arterial compliance: A systematic review and meta-analysis

To the Editor,

We read with interest the comprehensive meta-analysis of the effect of weight loss induced by energy restriction, with and without adjunctive therapies, on various measures of arterial compliance, by Petersen and colleagues published in *Atherosclerosis* [1].

Overall, modest weight loss was associated with some improvement in the cardio-ankle vascular index, β-stiffness index, arterial compliance and distensibility, distal oscillatory compliance, proximal capacitance compliance, systemic arterial compliance and reflection time. There was no significant effect on augmentation index, augmentation pressure, pulse pressure or strain.

We appreciate the varied nature of the literature in this area, which means that studies of different weight loss strategies (for example, diet only, diet and exercise, weight loss drugs or bariatric surgery) were grouped together to increase statistical power. However, in an attempt to explore this obvious source of heterogeneity, the authors conducted a series of sub-analyses in which the studies in each outcome were stratified by intervention type.

For a number of the outcomes explored (distensibility, distal oscillatory compliance and proximal capacitance compliance), the effect of combined diet and exercise-induced weight loss was associated with improvements in these markers, whereas a diet-only approach showed no significant improvement.

We would be interested in the Author's opinions on the impact exercise is having on these measures of arterial stiffness, as in another sub-analysis, combined diet and exercise produced an even stronger reduction in pulse pressure (-0.73 [-0.99, -0.47]) compared to diet alone (-0.47 [-0.82, -0.11]), suggestive of an additive effect of exercise and that it could possibly be potentiating the beneficial effects of diet on weight loss.

A Japanese study demonstrated that the change in cardio-ankle vascular index did not correlate with the change in BMI [2].

Furthermore, in Peterson and colleagues' study, obesity was considered using only the crude measure of body mass index, which may not accurately reflect adiposity, especially in older populations; and a previous review determined that body mass index was not associated with arterial stiffness [3].

Indeed, in the Japanese study, changes in CAVI were significantly related to changes in visceral adipose tissue area and not crude body weight measures [2]. Taken together, these data support the hypothesis that exercise should be incorporated into weight loss programmes and would probably enhance the effect of weight loss on arterial stiffness.

Exercise has proven benefits in reducing vascular risk [4]. A longitudinal study in Switzerland demonstrated that maintaining even moderate levels of physical activity was associated with reduced arterial stiffness in older adults [5]. Exercise, particularly aerobic, has numerous endothelial functions, including augmenting NO-dependent vasodilation, which affects arterial function [6,7]. As the authors note, this study serves as hypothesis generating material. It highlights the need to design targeted approaches to weight loss and further highlights the need for further research in well-designed randomised trials to elucidate critical findings lost in aggregated analysis.

Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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21 June 2016
Available online 30 June 2016

Appendix 7:

Rodríguez AJ, Ebeling P, Scott D. Sarcopenia and physical activity in older Australians. Australas Epidemiol 2015; 22: 11.

Round Table

Sarcopenia and physical activity in older Australians

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Originally, the term sarcopenia (derived from the Greek *sarx* and *penia* meaning 'lack of flesh') was proposed to describe the age-related loss of skeletal muscle mass[1] thought to contribute substantially to functional decline. However, research over the past 25 years has demonstrated that muscle mass is a relatively poor predictor of functional decline compared with muscle strength[2], and that muscle mass decline during aging may explain less than 10% of strength loss.[3] Disparities in the pathophysiology of muscle mass and strength decline have led some to propose a separate term for muscle strength declines ('dynapenia')[4], but in general, sarcopenia is now considered as age-related loss of muscle function in addition to muscle mass. The most widely accepted recent definition was developed by the European Working Group on Sarcopenia in Older People (EWGSOP) which describes sarcopenia as low muscle mass combined with low handgrip strength and/or gait speed.[5]

Recommended measurements for muscle mass quantification include appendicular lean mass by dual-energy X-ray absorptiometry (DXA), bioelectrical impedance analysis (BIA), computed tomography (CT) or magnetic resonance imaging (MRI).[6] Common muscle strength and physical performance assessments include handgrip and knee extension strength testing, gait speed and the Short Physical Performance battery (SPPB). However, there is no international consensus on the appropriate combination or cut-points for these measurements, and the lack of standardised methodologies makes sarcopenia prevalence estimation difficult.[5] Indeed, Bijlsma and colleagues reported a prevalence range of 0 to 45% in adults over the age of 60 years according to different definitions of low appendicular lean mass (normalised to height or body mass) and handgrip strength[7]. Similar controversy exists for sarcopenia prevalence using more recent multi-dimensional definitions; the EWGSOP estimate prevalence to range from 1–29% and 14–33% in community-dwelling and acute or long-term care populations, respectively.[5]

While prevalence estimates for sarcopenia vary substantially, loss of muscle mass and function is essentially ubiquitous in aging populations. *Figure 1* demonstrates that even in elite Australian weightlifters, peak muscle strength declines considerably from around 40 years of age. It is notable that

muscle strength declines exceeds mass declines by two-to-five-fold during aging.[3] This excess strength loss is attributed to the loss of *muscle quality,* including age-related neurological and skeletal muscle composition changes such as decreased voluntary activation of motor units and muscle fibre contractility. Many factors are thought to contribute to age-related declines in muscle mass and quality. Cellular and molecular triggers of sarcopenia include myocyte apoptosis, alterations in muscle protein turnover and impaired satellite cell function and regeneration.[8] These triggers are responsible for oxidative stress, mitochondrial dysfunction, inflammation, hormonal changes, fibre disorganisation and neuromuscular imbalances, which can lead to the preferential loss of fast motor units.[9] Aging is also associated with preferential loss and atrophy of type II muscle fibres which compromises force production capability.[9,10] Fat tissue increasingly infiltrates muscle (inter/intra-muscular adipose tissue; IMAT) reducing the net amount of available contractile muscle tissue. A higher amount of IMAT therefore increases the load burden of remaining muscle and is independently associated with poor physical performance in older adults.[1]

Figure 1. Combined Australian records ('clean and jerk' plus 'snatch') for Masters' weightlifting athletes according to age category. Data obtained from www.awf.com.au/resultsrankings/rptrecords.aspx on 23/04/2015.
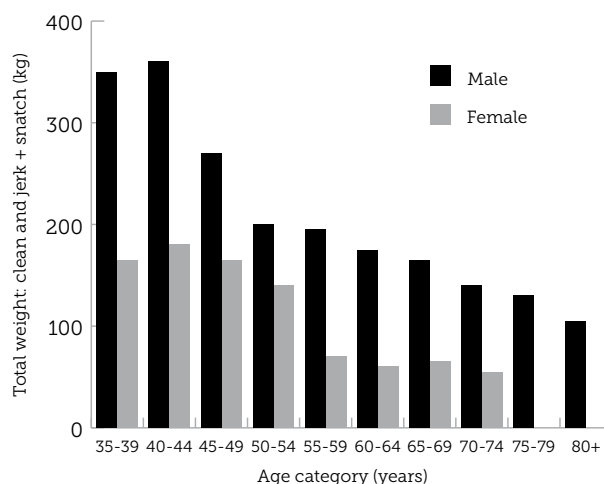
The consequences of sarcopenia include mobility limitation, disability and increased risk of falls and fractures.[12] These outcomes contribute substantially to acute and long-term healthcare costs. Direct costs attributable to sarcopenia in the US were estimated at $18.5 billion in 2000, representing about 1.5% of total healthcare expenditure at that time.[13] It was estimated that a 10% reduction in sarcopenia prevalence would result in savings of $1.1 billion per year. Although cost estimates for the Australian population are not available, increasing life expectancy indicates that sarcopenia is a growing economic burden. Interventions which can reduce sarcopenia prevalence will be vital in enabling older Australians to maintain independence and quality of life, with subsequent economic and societal benefits.

Maintaining and increasing physical activity (PA) is the primary strategy for preventing and reversing sarcopenia in older adults. In an older Japanese population, the odds of sarcopenia amongst individuals in the lower quintile of activity (walking less than 3,000–3,400 steps or performing less than 6–7 minutes of moderate intensity activity per day) was 2–4.5-fold greater than individuals in the upper quintile of activity (10,000–10,500 steps and 31–34 minutes of activity).[14] Similarly in an older community-dwelling Australian population, pedometer-determined steps per day was positively associated with maintenance of lean leg mass in both sexes, and leg strength and muscle quality in women, over three years.[15] Additionally, older adults who achieved ≥10,000 steps/day had significantly greater leg strength and muscle quality compared to those who did not.[16] In a separate Australian study of older adults aged 60 to 86 years, each hour of sedentary time was associated with a 33% increased likelihood of sarcopenia defined as low appendicular lean mass plus low muscle strength or physical performance, independent of PA levels.[17] From a public health perspective, it is important to promote a lifecourse approach to PA because high levels of PA in childhood and adolescence will maximise the lifetime peak in muscle strength, while maintenance of PA through adulthood and older age will reduce the rate of strength decline, and therefore risk for disability (*Figure 2*).

Figure 2: Muscle strength throughout the life course for physically active and inactive individuals.
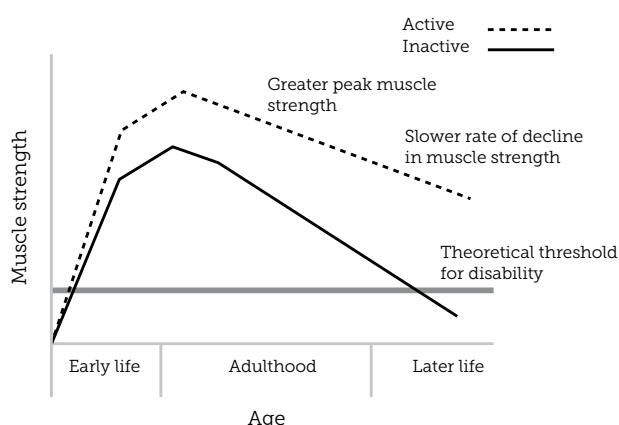
Resistance training (e.g. weightlifting) appears to be the most effective mode of PA for reversing sarcopenia, and can be safely performed in frail elderly people.[18, 19] Randomised controlled trials of resistance training alone, and combined with endurance training, have demonstrated significant improvements in muscle mass and function (knee extension power and strength) and physical performance (chair rise, stair climb and 12–min walk tests) in older adults.[20-22] In particular, high-velocity progressive resistance training, involving rapid concentric muscle contractions, is recommended given that it is effective for improving muscle mass, strength, power and bone mineral density in older adults.[23] Although evidence is unequivocal that exercise involving a resistance training component can translate into improvements in sarcopenia outcomes in the majority of older adults, some older adults demonstrate poor responsiveness to exercise. In a study of overweight or obese older individuals (65–79yo), 30% of participants who underwent five months of resistance training showed no change, or even decreases, in strength[24], with results indicating that high levels of initial adiposity explained blunted responses to exercise. Protein and vitamin D supplementation may have promise in enhancing the effects of exercise for sarcopenia, and it is possible that such adjuvant therapies can be of benefit particularly in older adults at risk of poor exercise responsiveness.[25]

The recommendations for older adults provided in the Australian Government's *Be active for life* campaign[26] generally promote activities beneficial to sarcopenia, including completing a range of physical activities that incorporate fitness, strength, balance and flexibility and accumulating at least 30 minutes of moderate intensity physical activity on most, preferably all, days. However, the Australian Bureau of Statistics reports that less than 15% of men and women aged 65–74yo and less than 5% of men and women aged 75yo or older achieve the PA recommendation of 10,000 steps per day.[27] Recent literature has identified poor health, lack of interest, lack of access and lack of safety as barriers to PA in older people (65–100yo).[28] Given that each of these barriers is to some extent modifiable, there is significant potential to encourage reduced sedentary time and increased PA in older Australians.[17] An emerging option for increasing PA interest through providing self-motivation and self-monitoring is wearable technologies.[29] Increasingly affordable devices exist that enable simple and real-time performance tracking and feedback for a growing number of measurements including steps, elevation gain, calories, heart rate and intensity of activity.[30] In addition, many devices may be linked with smartphone applications. These devices and apps have the potential to provide a sense of empowerment regarding personal health and lead to sustained behavioural changes.[31] Furthermore, data could be utilised by doctors, physiotherapists, or exercise physiologists to remotely assess a patient's progress towards PA goals. Nevertheless, future research is required to understand whether these devices can be effectively implemented into exercise programs for older adults.

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Australia's aging population presents significant challenges for policymakers, health practitioners and the wider community. Sarcopenia will make a substantial contribution to increasing demand for disability support (including pensions), and health and aged care access, in coming years. The lack of standardised criteria for sarcopenia is a persistent source of confusion for practitioners and it is incumbent on researchers to establish a consensus operational definition and promote clinical recognition of sarcopenia as a health disorder. At the policy level, a focus on a life course approach to PA for sarcopenia in the general population, and improving access to evidence-based exercise interventions for middle-aged and older adults, have the potential to significantly reduce the disease burden of sarcopenia.

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Appendix 8:

Morton SK*, Rodríguez AJ*, Morris DR, et al (2016) "A

Systematic Review and Meta-Analysis of Circulating

Biomarkers Associated with Failure of Arteriovenous

Fistulae for Haemodialysis" PLoS One 11:e0159963. doi:

10.1371/journal.pone.0159963

RESEARCH ARTICLE

A Systematic Review and Meta-Analysis of Circulating Biomarkers Associated with Failure of Arteriovenous Fistulae for Haemodialysis

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Data Availability Statement: All relevant data are within the paper and its supporting information files.

Abstract

Background

Arteriovenous fistula (AVF) failure is a significant cause of morbidity and expense in patients on maintenance haemodialysis (HD). Circulating biomarkers could be valuable in detecting patients at risk of AVF failure and may identify targets to improve AVF outcome. Currently there is little consensus on the relationship between circulating biomarkers and AVF failure. The aim of this systematic review was to identify circulating biomarkers associated with AVF failure.

Methods

Studies evaluating the association between circulating biomarkers and the presence or risk of AVF failure were systematically identified from the MEDLINE, EMBASE and Cochrane Library databases. No restrictions on the type of study were imposed. Concentrations of circulating biomarkers of routine HD patients with and without AVF failure were recorded and meta-analyses were performed on biomarkers that were assessed in three or more studies with a composite population of at least 100 participants. Biomarker concentrations were synthesized into inverse-variance random-effects models to calculate standardized mean differences (SMD) and 95% confidence intervals (CI).

Results

Thirteen studies comprising a combined population of 1512 participants were included after screening 2835 unique abstracts. These studies collectively investigated 48 biomarkers, predominantly circulating molecules which were assessed as part of routine clinical care. Meta-analysis was performed on twelve eligible biomarkers. No significant association between any of the assessed biomarkers and AVF failure was observed.

Conclusion

This paper is the first systematic review of biomarkers associated with AVF failure. Our results suggest that blood markers currently assessed do not identify an at-risk AVF. Further, rigorously designed studies assessing biological plausible biomarkers are needed to clarify whether assessment of circulating markers can be of any clinical value. PROSPERO registration number CRD42016033845.

Introduction

A surgically created arteriovenous fistula (AVF) is the preferred form of long term vascular access (VA) for use in haemodialysis (HD) therapy. Recent statistics from the USA show that in 2015 66% of routine HD patients used an AVF for VA, and this proportion is predicted to increase in line with the national 'Fistula First Catheter Last' initiative [1]. Nevertheless, AVF failure resulting from complications such as venous stenosis and thrombosis remain a major cause of hospitalization and morbidity within the HD population [2]. The Dialysis Outcomes Quality Initiative (DOQI) has reported that primary AVF failure is approximately 15% after one year and 25% after two years [3]. Recent data suggest that AVF survival has not significantly improved more than a decade after standardised VA guidelines were introduced [4].

Currently AVFs are monitored using duplex ultrasound (US) to assess blood flow and identify flow disturbances in the AVF or adjacent vessels. While routine screening may improve AVF survival rates by allowing early identification and remediation of at-risk fistulae, US-based screening programs are time and labour intensive and rely on specialist equipment which is often unavailable at regional centres [5]. In contrast, screening programs based on the detection of blood-borne markers (biomarkers) could provide a more cost effective means to identify patients at risk of AVF failure.

Few studies have assessed the relationship between biomarkers and AVF failure, and discrepancy exists between the conclusions reported. To date, there has been no systematic evaluation of the available literature to clarify the reported association between circulating biomarkers and AVF failure. Accordingly, we performed a systematic review and meta-analysis of publicly available literature to examine the association of circulating biomarkers with AVF failure.

Methods

Search protocol and study focus

We performed a systematic literature review of published work in accordance with the MOOSE guidelines [6]. This review was registered in the PROSPERO International Registry, registration number CRD42016033845. We sought studies that investigated the association of at least one circulating biomarker with an AVF outcome (such as thrombosis, stenosis or failure) in patients receiving regular HD. We predominantly sought literature from the online MEDLINE (January 1966 to December 2015), EMBASE (January 1980 to December 2015) and the Cochrane Library databases as well as scanning reference lists of studies captured in the literature search. In performing literature searches, we applied the search terms "AVF" AND "vascular access", as well as one of the following title/abstract phrases: "biomarker", "concentration", "function", "dysfunction", "maturation", "patency", "failure", "survival", "thrombo*", "steno*", "factor", "predict*", "serum", "plasma", "circulating", "risk factor" and "blood" with

no language restriction (See S1 File for details). Titles and abstracts of identified searches were screened and if the suitability of the article was uncertain, the full text was assessed. We considered a native AVF to mean the anastomosis of an artery and vein; and graft to mean the surgical placement of a loop or bypass (either from autologous tissue or synthetic material) to join an artery and vein. AVF failure was defined as complications in the VA which prevented successful HD, arising from events such as AVF stenosis or thrombosis.

Study eligibility

Studies were deemed eligible if i) the patient population investigated were using, or were to receive a native AVF for HD; ii) the study assessed and reported the association of circulating biomarker(s) with the presence or risk of AVF failure; iii) cases were patients with a malfunctioning AVF from any reason (defined as AVF failure) and controls were patients whose AVF remained functioning and able to be used for HD (defined as a patent AVF); iv) specific details of the timing of blood collection relative to AVF failure were provided, and v) the full manuscript was in English. Specific exclusion criteria included i) animal model studies; ii) studies investigating non-surgically created AVFs; iii) non-HD related AVFs; and iv) studies evaluating multiple types of VA without providing AVF-specific results.

Quality assessment, data extraction and biomarker selection for meta-analysis

Data extraction was performed using a standardised data extraction form (S2 File). The following clinical data were extracted from all studies: 1) General patient characteristics (e.g. age, sex and smoking); 2) Definitions of case and control groups; 3) Definitions of patent or failed AVF; 4) Timing of blood sampling relative to AVF failure, and blood medium assessed; 5) Outcome measures; 6) Methods of biomarker quantification; 7) Statistical analyses performed, including reported concentrations, effect estimates, variability, and p-values. The type of study design was also recorded (e.g. cohort or case control). Each study was assessed using a modified version of the Ottawa-Newcastle tool to assess the risk of bias. The assessment tools and subsequent results are provided within the supplementary material (S3 and S4 Files for cohort and case control studies, respectively). Risk of bias was classified very low, low, medium, high or very high, depending on the assessment outcome (see S3 and S4 Files for specific details regarding cohort and case control studies, respectively).

Biomarkers which were assessed by $\geq$3 independent studies in a composite population of $\geq$100 patients were included in a meta-analysis. Results of the meta-analysis are presented as mean and standard deviation (SD). Where studies presented data as mean and standard error of the mean (SEM), standard deviation was recalculated using the following equation: $SD = SEM \times \sqrt{n}$, where n = population size.

Meta-analysis

Biomarker concentrations were compared between patients with (cases), and without (controls) AVF failure. An inverse-variance random-effects model was applied to determine the standardised mean difference (SMD) and 95% confidence intervals (95% CI) of biomarker concentrations between case and control groups. Inter-study heterogeneity was determined using the I^2 index and its associated p-value, as detailed by detailed by Higgins *et al* [7]. All statistical analyses were performed using RevMan v5.3 software (The Nordic Cochrane Centre, The Cochrane Collaboration, 2014) and GraphPad Prism v6.05 software. For all reported comparisons p-values <0.05 were considered significant.

Results

Literature search

Initial database searches yielded 4235 potentially eligible papers for inclusion. A further nine studies were identified from eligible reference lists (Fig 1). After removing 1409 duplicates, 2835 unique abstracts were screened. Of these, 2756 were excluded, mainly because they did not assess a circulating biomarker, and the full text of 79 studies were assessed. Sixty six studies were excluded after reviewing the full text, primarily due to failure to specify the timing of blood collection relative to AVF failure. A total of thirteen studies satisfied the eligibility criteria and were included in this review (Fig 1) [8–20].

Study characteristics and risk of bias

The characteristics of the included studies are summarised in Table 1. Nine of the studies adopted a longitudinal cohort design (8 prospective [8, 10, 12–14, 17, 19, 20] and 1 retrospective [16]), the remaining four were cross-sectional case-control studies [9, 11, 15, 18]. Of the thirteen studies, only three adjusted their results for the VA risk factors of age and sex [10, 16,
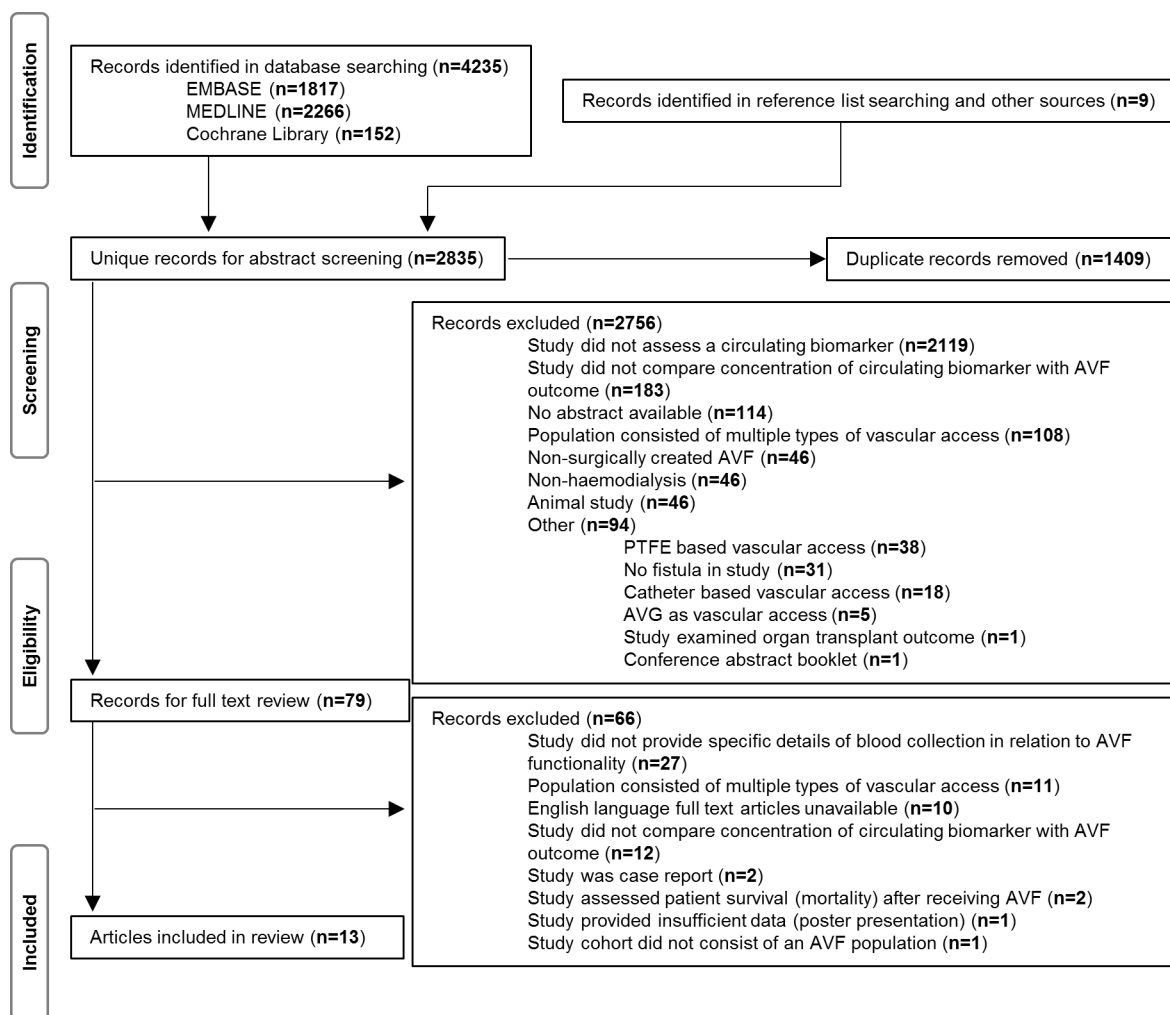
Fig 1. PRISMA study selection flow chart.

doi:10.1371/journal.pone.0159963.g001

Table 1. Characteristics of the included studies.

Reference	No. Patients	Study Location	Study Design	AVF failure due to	Method(s) of diagnosis of AVF failure	Time of Blood Collection	History of AVF functionality	AVF Location (Case vs Control)
Baumann 2003 [8]	N = 62 Cases:24 Controls:38	Germany	Cohort	Thrombosis within 30 days of AVF creation	Ultrasound and/or Surgery	Before AVF creation	Newly created AVF	n/r
Bilgic 2015 [9]	N = 94 Cases:51 Controls:43	Turkey	Case Control	Stenosis	Ultrasound and Fistulogram	At AVF failure	Functional for at least 6 months prior to analysis (cases and controls)	n/r
Bojakowski 2012 [10]	N = 45 Cases:11 Controls:34	Poland	Cohort	Stenosis and/or thrombosis within 12 weeks of AVF creation	Ultrasound and Angiography	At AVF creation	Newly created AVF. AVF patent for 52 weeks post creation (controls)	RC–(100% vs 100%)
Candan 2014 [11]	N = 80 Cases:42 Controls:38	Turkey	Case Control	Thrombosis	Ultrasound and/or Fistulogram	Collected prior to a mid-week dialysis treatment	Newly created AVF, functional for 3 months prior to failure (cases). Functional AVF for over 3 years post creation (controls)	n/r
Gagliardi 2011 [12]	N = 91 Cases:37 Controls:54	Italy	Cohort	Thrombosis	Access blood flow monitoring	Collected at monthly intervals	Functional with no pre-existing vascular abnormalities (cases and controls)	BC–(100% vs 100%)
Jaberi 2007 [13]	N = 58 Cases:18 Controls:40	Canada	Cohort	Cephalic arch stenosis	Fistulogram	Variable times within 6 months of failure diagnosis	Unclear	BC–(94% vs 70%)
Kaygin 2013 [14]	N = 386 Cases:75 Controls:311	Turkey	Cohort	Failure to mature	Dialysis complications	At AVF creation	AVF failure within first 12 weeks (cases). AVF patent at end of 12 weeks (control)	RC–(59% vs 69%) BC–(41% vs 30%)
Kim 2013 [15]	N = 64 Cases:34 Controls:30	Korea	Case Control	Stenosis	Ultrasound	Prior to midweek dialysis at monthly intervals for a total of 6 months	Functional with no pre-existing abnormalities for at least 6 months (cases and controls)	RC–(100% vs 100%)
Kirkpantur 2008 [16]	N = 99 Cases:38 Controls:61	Turkey	Cohort	Thrombosis	Dialysis complicationsand Angiography	At AVF creation plus fasting monthly pre-HD collections up to failure (cases) or end of follow up (controls)	AVF were patent for at least 6 weeks following surgical opening of AVF (cases and controls)	RC–(76% vs 77%) BC–(24% vs 21%)
Masaki 1999 [17]	N = 184 Cases:83 Controls:101	Japan	Cohort	Stenosis or Thrombosis	Surgery and/or Radiography and/or Ultrasound	At AVF failure (cases), unspecified for controls	n/r	n/r
Ozdemir 2005 [18]	N = 141 Cases:60 Controls:81	Turkey	Case Control	Thrombosis	n/r	6 months prior to AVF thrombosis (cases) or 6 months prior to study (controls)	One or more thromboses (cases). No recorded thromboses (controls)	SB–(30.8% vs 4.9%)
Wu 2009 [19]	N = 100 Cases:41 Controls:59	China	Cohort	Restenosis following PTA	Fistulography	Immediately before PTA (cases) or routine HD (controls)	All patients had history of stenosis (cases and controls) and underwent PTA	n/r
Yilmaz 2014 [20]	N = 108 Cases:64 Controls:44	Turkey	Cohort	Stenosis	Ultrasound and Angiography	Measured 6 months prior to stenosis diagnosis	Functional for at least 6 months prior to analysis (cases and controls)	n/r

AVF: arteriovenous fistula; n/r: not recorded; HD: haemodialysis; RC: radiocephalic AVF; BC: brachiocephalic AVF. SB: Snuffbox AVF; PTA: percutaneous transluminal angioplasty. Studies by Baumann *et al*. and Masaki *et al*. were not included in the meta-analysis as neither study presented the mean concentration of measured biomarker(s) within the text.

19]. Only five studies collected blood samples for biomarker measurement at a time considered appropriate to the primary outcome assessed, i.e. for studies assessing the future risk of AVF complications, blood collection within one week of AVF creation was deemed appropriate, and for studies assessing biomarkers associated with the presence of an AVF complication, blood collection within one week of the time of AVF failure [8–10, 14, 19]. All but four studies included an AVF population representative of the general HD population (e.g. excluded co-morbidities such as active infection) [8, 12, 18, 19], and only one failed to provide clear patient selection criteria [17].

Using the modified Ottawa-Newcastle tool to assess the risk of bias in the nine cohort studies (S3 File) we found one with a very low risk [10], 3 studies with a low risk [8, 12, 14], 2 studies with a medium risk [16, 17] and the final 3 with a high risk of bias [13, 19, 20]. Of the case control studies, 2 were considered low risk [9, 11] and 2 were considered medium risk [15, 18] of bias (S4 File).

The combined population of the thirteen studies totalled 1512 participants, including 578 cases and 934 controls. Total sample size ranged from 45 to 386 patients [10, 14]. The median number of cases per study was 40 (range 11 to 83), with a median age of 57 years (range 45 to 68) and comprising a median 55.8% males (range 36.6 to 65.2). The median number of controls per study was 44 (range 30 to 311), with a median age of 55 years (range 41 to 62) and comprising a median 56.7% males (range 45.8 to 70.6).

All studies, excluding that of Baumann *et al*, and Masaki *et al*., listed mean biomarker concentrations for groups of patients with failed (cases) or patent (controls) AVFs [8, 17]. The study by Bojakowski *et al*. compared groups of patients who had early fistula failure (12 weeks post AVF creation), late fistula failure (52 weeks post AVF creation) or no fistula failure [10]. For the purposes of this review, patients with early fistula failure were considered to be the cases as failure occurred closer to the time of blood collection (at time of AVF creation), and patients with late AVF failure were excluded [10]. Five studies included only patients with a newly formed fistula in anticipation of HD [8, 10, 11, 14, 16], while the remaining studies included only patients receiving routine HD [9, 12, 13, 15, 17–20]. In all but one study, control patients included in this review had an AVF that was patent for at least 6 months. In contrast, all patients included in the study by Wu *et al*., had received a percutaneous transluminal angioplasty (PTA) to resolve a previous AVF dysfunction and controls were considered those patients who did not experience restenosis following this procedure [19].

Reported biomarkers and AVF failure

We observed considerable inter-study variation in the definition of AVF failure. For the purpose of this meta-analysis we initially included all outcomes reported (stenosis, thrombosis and AVF dysfunction arising from unknown complications which led to HD complications) as AVF failure (Table 1). Six studies investigated the association of circulating biomarkers with AVF stenosis or restenosis [9, 13, 15, 16, 19, 20] four studies with AVF thrombosis [8, 11, 12, 18], two with a mixture of both AVF stenosis and thrombosis [10, 17], and one study did not specify the cause of AVF dysfunction [14]. There was considerable disparity in the timing of blood sample collection relative to AVF failure between studies (Table 1). Three studies provided mean biomarker values over time, often including samples taken at the time of AVF creation, as well as samples leading up to and including AVF dysfunction [12, 15, 16]. Three studies analysed blood samples before or at the time of fistula creation only [8, 10, 15], and four at the time of AVF failure only [9, 11, 17, 19]. The final three studies collected blood samples within the 6 months prior to AVF failure [13, 18, 20]. Most studies lacked specific details of the methods of biomarker measurement, with many stating quantification was achieved by an "automated analyser" (S1 Table). Furthermore it was not well reported whether biomarkers

were measured in plasma, serum or whole blood (S1 Table). For the purpose of the meta-analysis, biomarker data from only 11 of the thirteen studies were included as Baumann *et al* and Masaki *et al* did not provide mean biomarker concentrations for patients that did and did not have AVF failure [8, 17].

Comparison of biomarker concentrations in cases and controls

All biomarkers assessed in the reviewed studies are shown in S1 Table. Out of the 48 measured biomarkers, 12 (albumin, creatinine, C-reactive protein (CRP), calcium, ferritin, haemoglobin, high-density lipoprotein (HDL-C) cholesterol, low-density lipoprotein (LDL-C) cholesterol, parathyroid hormone, phosphorus, total cholesterol (TC) and triglycerides (Table 2)) satisfied the inclusion criteria for the current meta-analysis. The number of studies assessing each biomarker varied from 10 studies (albumin) to three studies (creatinine). Mean concentrations of the biomarkers in case and control groups reported by each study are listed in Table 3. There was considerable intra- and inter-study variation in the number of decimal places reported for each biomarker concentration and thus all biomarkers are listed to the decimal point as originally published. Further, for all but one paper (Jaberi *et al.*, [13]), biomarker concentrations were reported in United States standard (US) units and so to avoid decimal point rounding errors, the subsequent meta-analyses were performed using the concentrations reported in US units. Data from Jaberi *et al.*, was converted from International Standard (SI) units into US units for inclusion into the meta-analysis [13]. For the purpose of clarity, biomarker concentrations are presented in both US and SI units in Table 3. It is important to note that due to the often interchangeable use of the term phosphorus and phosphate between studies, data relating to either terminology were included in the meta-analysis denoted here as 'phosphorus' [21].

Meta-analysis findings

The association of biomarkers with all AVF failure. The association of the eligible 12 biomarkers with AVF failure was assessed using an inverse-variance random-effects model. None of the twelve biomarkers were found to be significantly associated with AVF failure when we included all measured outcomes of AVF failure (Table 4, S1 Fig). A leave-one-out sensitivity analysis was performed for each of the twelve biomarkers (S5 File). Only the exclusion of the data by Kirkpantur *et al.* led to statistically significant inverse associations of elevated

Table 2. Biomarkers for which meta-analyses were performed.

Biomarker	No. of studies	References	Total population
Albumin	10	9–16, 19, 20	1125
Calcium	5	9, 13, 15, 19, 20	424
Creatinine	3	10, 14, 19	531
CRP	9	9–12, 14, 15, 18–20	1109
Ferritin	5	9–11, 18, 20	468
Haemoglobin	6	9–11, 13, 16, 20	484
HDL-C	7	9–11, 14, 16, 19, 20	912
LDL-C	7	9–11, 14, 16, 19, 20	912
PTH	4	9, 11, 18, 20	423
Phosphorus	5	9, 13, 15, 19, 20	424
Total cholesterol	4	11, 12, 14, 16	656
Triglycerides	7	9–11, 14, 16, 19, 20	912

CRP: C-reactive peptide; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol; PTH: Parathyroid hormone.

doi:10.1371/journal.pone.0159963.t002

Table 3. Biomarker concentrations in patients with (case) and without (control) AVF failure

Marker US units (SI units)	ref	Cases			Controls			p
		n	Mean	SD	n	Mean	SD	
Albumin g/dL (g/L)	9	51	3.87 (38.7)	0.36 (3.6)	43	3.91 (39.1)	0.42 (4.2)	0.189
	10	11	3.2 (32)	1.1 (11)	34	4.0 (40)	0.4 (4)	dns
	11	42	3.8 (38)	0.3 (3)	38	3.8 (38)	0.3 (3)	0.924
	12	37	3.26 (32.6)	0.40 (4.0)	54	3.49 (34.9)	0.46 (4.6)	**0.018**
	13	18	3.4 (34)	0.4 (4)	40	3.3 (33)	0.4 (4)	n/r
	14	75	3.0 (30)	0.8 (8)	311	3.96 (39.6)	0.4 (4)	**<0.001**
	15	34	3.8 (38)	3.5 (35)	30	3.9 (39)	2.2 (22)	0.761
	16	38	3.67 (36.7)	0.26 (2.6)	61	3.95 (39.5)	0.39 (3.9)	**0.010**
	19[1]	41	3.59 (35.9)	0.44 (4.4)	59	3.63 (36.3)	0.43 (4.3)	0.64
	20	64	3.76 (37.6)	0.68 (6.8)	44	3.73 (37.3)	0.56 (5.6)	**0.005**
Calcium mg/dL (mmol/L)	9	51	8.40 (2.10)	0.52 (0.13)	43	8.50 (2.13)	0.65 (0.16)	0.231
	13	18	9.16 (2.29)	0.60 (0.15)	40	9.08 (2.27)	0.72 (0.18)	n/r
	15	34	8.5 (2.1)	4.1 (1.0)	30	8.6 (2.2)	2.7 (0.7)	0.719
	19	41	9.97 (2.49)	1.04 (0.26)	59	9.89 (2.47)	0.86 (0.21)	0.66
	20	64	8.02 (2.00)	0.64 (0.16)	44	8.10 (2.02)	0.55 (0.14)	0.422
Creatinine mg/dL (μmol/L)	10	11	5.2 (459.7)	1.9 (168.0)	34	5.1 (450.8)	1.9 (168.0)	dns
	14	75	4.2 (371.3)	2.6 (229.8)	311	4.1 (362.4)	2.5 (221.0)	0.597
	19	41	10.60 (937.04)	2.20 (194.48)	59	10.20 (901.68)	2.20 (194.48)	0.34
CRP mg/L (nmol/L)	9	51	18.77 (178.77)	20.48 (195.05)	43	13.28 (126.48)	12.47 (118.76)	0.271
	10	11	18.6 (177.15)	16.8 (160.0)	34	7.3 (69.5)	6.6 (62.9)	dns
	11	42	12.6 (120.0)	16.6 (158.1)	38	12.4 (118.1)	16.3 (155.24)	0.940
	12[2]	37	11.98 (114.10)	9.1 (86.7)	54	9.83 (93.62)	11.4 (108.6)	0.341
	14	75	18.6 (177.2)	4.3 (41.0)	311	4.6 (43.8)	2.2 (21.0)	**<0.001**
	15	34	3.8 (36.2)	13.4 (127.6)	30	4.0 (38.1)	15.3 (145.72)	0.479
	18	60	12.9 (122.9)	15.0 (142.9)	81	11.2 (106.7)	11.4 (108.6)	n/r
	19	41	7.3 (69.5)	9.1 (86.7)	59	8.8 (83.8)	10.0 (95.2)	0.44
	20	64	9.75 (92.86)	11.97 (114.00)	44	8.94 (85.1)	12.3 (117.2)	0.502
Ferritin ng/mL (pmol/L)	9	51	422.4 (949.1)	240.8 (541.1)	43	439.1 (986.7)	230.7 (518.4)	0.346
	10	11	170.4 (382.9)	104.7 (235.3)	34	235.7 (529.6)	314.5 (706.7)	dns
	11	42	855.1 (1921.4)	714.9 (1606.4)	38	890.6 (2001.2)	619.1 (1391.1)	0.814
	18	60	552.4 (1241.2)	821.6 (1846.1)	81	497.6 (1118.1)	308.4 (693.0)	n/r
	20	64	542.43 (1218.77)	230.45 (517.8)	44	539.15 (1211.47)	286.37 (643.47)	0.657
Hb g/dL (g/L)	9	51	10.85 (108.5)	1.15 (11.5)	43	10.90 (109.0)	1.26 (12.6)	0.542
	10	11	9.7 (97)	1.0 (10)	34	10.9 (109)	1.5 (15)	dns
	11	42	11.6 (116)	1.5 (15)	38	11.3 (113)	1.3 (13)	0.266
	13	18	11.6 (116)	1.5 (15)	40	11.6 (116)	1.3 (13)	n/r
	16	38	10.9 (109)	1.0 (10)	61	11.2 (112)	1.0 (10)	0.080
	20	64	10.83 (108.3)	1.97 (19.7)	44	10.75 (107.5)	1.82 (18.2)	0.848
HDL-C mg/dL (mmol/L)	9	51	39.6 (1.0)	10.3 (0.3)	43	38.7 (1.0)	11.2 (0.3)	0.305
	10	11	52.1 (1.4)	18.5 (0.5)	34	56.7 (1.5)	17.6 (0.5)	dns
	11	42	33.9 (0.9)	13 (0.3)	38	32.4 (0.8)	8.9 (0.2)	0.552
	14	75	42.8 (1.1)	12.5 (0.32)	311	39.6 (1.0)	11.8 (0.3)	n/r
	16	38	31.4 (0.8)	4.4 (0.1)	61	44 (1.1)	7 (0.2)	**0.015**
	19	41	54 (1.4)	19 (0.5)	59	50 (1.3)	17 (0.4)	0.24
	20	64	31.8 (0.8)	12.6 (0.3)	44	51.5 (1.3)	11.9 (0.3)	**<0.001**

(*Continued*)

Table 3. (*Continued*)

Marker US units (SI units)	ref	Cases			Controls			*p*
		n	Mean	SD	n	Mean	SD	
LDL-C mg/dL (mmol/L)	9	51	154.5 (4.0)	32.6 (0.8)	43	128.7 (3.3)	28.6 (0.74)	**<0.001**
	10	11	108.6 (2.8)	48.1 (1.3)	34	99.5 (2.6)	45.7 (1.2)	dns
	11	42	98 (2.5)	35.1 (0.9)	38	95.9 (2.5)	33 (0.9)	0.784
	14	75	118.7 (3.1)	28.6 (0.7)	311	114.8 (3.0)	28.3 (0.7)	n/r
	16	38	62.8 (1.6)	11.0 (0.3)	61	97.4 (2.5)	19 (0.5)	**0.022**
	19	41	113 (2.9)	33 (0.9)	59	102 (2.6)	30 (0.8)	0.09
	20	64	102.69 (2.66)	36.13 (0.94)	44	99.86 (2.59)	39.49 (1.02)	0.378
PTH pg/mL (= ng/L)	9	51	332.8	160.5	43	319.5	204.3	0.105
	11[3]	42	267.5	229.5	38	311.4	316.1	0.477
	18	60	449.4	363.4	81	492.0	409.9	n/r
	20	64	371.70	301.04	44	361.57	327.48	0.815
Phosphorus mg/dL (mmol/L)	9	51	5.80 (1.87)	1.90 (0.61)	43	5.75 (1.86)	1.79 (0.58)	0.365
	13[4]	18	6.25 (2.02)	2.20 (0.71)	40	5.14 (1.66)	1.46 (0.47)	n/r
	15	34	5.3 (1.7)	26.2 (8.46)	30	5.2 (1.7)	26.8 (8.7)	0.813
	19[4]	41	4.39 (1.42)	1.36 (0.44)	59	4.45 (1.44)	1.74 (0.56)	0.84
	20	64	6.29 (2.03)	1.51 (0.49)	44	6.13 (1.98)	1.49 (0.48)	0.375
TC mg/dL (mmol/L)	11	42	167 (4.3)	45.3 (1.2)	38	160.7 (4.2)	43.7 (1.1)	0.544
	12	37	148.24 (3.84)	23.50 (0.61)	54	136.00 (3.52)	35.60 (0.92)	0.069
	14	75	183.5 (4.8)	28.2 (0.7)	311	172.3 (4.5)	44.9 (1.2)	n/r
	16	38	145.6 (3.8)	35.1 (0.9)	61	155 (4)	34.5 (0.9)	0.860
TG mg/dL (mmol/L)	9	51	279.5 (3.2)	90.4 (1.0)	43	288.9 (3.3)	106.6 (1.2)	0.411
	10	11	158.4 (1.8)	75.8 (0.9)	34	148.6 (1.7)	82.4 (0.9)	dns
	11	42	181.8 (2.1)	84.7 (1.0)	38	170.1 (1.9)	84.7 (1.0)	0.891
	14	75	148.6 (1.7)	74.8 (0.9)	311	153.8 (1.7)	82.2 (0.9)	n/r
	16	38	143.6 (1.6)	66.2 (0.8)	61	161.3 (1.8)	61.5 (0.7)	0.390
	19	41	175 (2.0)	105 (1.2)	59	155 (1.8)	80 (0.9)	0.27
	20	64	195.58 (2.2)	89.41 (1.0)	44	198.78 (2.3)	96.436 (1.09)	0.865

CRP: C-reactive peptide; Hb: Haemoglobin; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol; PTH: Parathyroid hormone; TC: Total cholesterol; TG: Triglycerides. There was considerable inter and intra-study variability in the number of decimal places reported for each biomarker observed and to avoid ambiguity, data are shown exactly as reported by the original studies. Biomarker concentrations are reported in both US and SI units (the latter in parentheses). P-values reported as published, those in bold are considered significant. Those p-values not reported denoted as n/r, those p-values not reported due to ANOVA analyses denoted as dns (did not specify). n = patient number; SD: standard deviation; AVF: arteriovenous fistula. Gagliardi *et al* [12] and Kaygin *et al* [14] did not specify SD or standard error of the mean (SEM), however based on reported p-values it was assumed to be SD. Kim *et al* [15] reported SEM, therefore SD was manually calculated using the formula: $SD = SEM \times \sqrt{n}$. [1]Values reported by [19] as mg/dL, however this would make the albumin values 1000-fold different from the other reported values, therefore assumed to be g/dL. [2]Values reported by [12] as ng/mL, however this would make the CRP values 1000-fold different from the other reported values, therefore assumed to be mg/L. [3]Values reported by [11] as pg/dL, however this would make the PTH values 100-fold different from the other reported values, therefore assumed to be pg/mL. [4]Values reported by [13] and [19] are reported as phosphate measurements, however the terms phosphorus and phosphate are often interchangeably used in clinical reports [21] and therefore all measurements were considered to be phosphorus.

doi:10.1371/journal.pone.0159963.t003

LDL-C and TC with AVF failure (p = 0.02 and p = 0.007 respectively) [16]. High I[2] values were observed for most meta-analyses when including all eligible studies ([Table 4](#)), with little change observed during most of the leave-one-out analyses ([S5 File](#)).

The association of biomarkers with AVF thrombosis or stenosis. Sub-analyses were conducted to specifically assess the association of biomarkers with AVF stenosis/restenosis [9,

Table 4. Meta-analysis of biomarker data in relation to AVF failure.

Biomarker	Studies	N_Cases	N_Controls	SMD (95%CI)	p value*	I²	I² p value
Albumin	10	411	714	-0.44 [-0.95, 0.07]	0.09	93%	**< 0.001**
Calcium	5	208	216	-0.04 [-0.24, 0.15]	0.67	0%	0.86
Creatinine	3	127	404	0.08 [-0.13, 0.28]	0.46	0%	0.84
CRP	9	415	694	0.75 [-0.32, 1.82]	0.17	98%	**< 0.001**
Ferritin	5	228	240	-0.01 [-0.19, 0.18]	0.92	0%	0.92
Haemoglobin	6	224	260	-0.10 [-0.33, 0.14]	0.42	36%	0.17
HDL-C	7	322	590	-0.45 [-1.12, 0.23]	0.20	95%	**< 0.001**
LDL-C	7	322	590	-0.06 [-0.64, 0.53]	0.85	93%	**< 0.001**
PTH	4	217	206	-0.04 [-0.23, 0.15]	0.67	0%	0.83
Phosphorus	5	208	216	0.10 [-0.10, 0.30]	0.32	4%	0.39
TC	4	192	464	0.14 [-0.12, 0.41]	0.28	50%	0.11
TG	7	322	590	-0.02 [-0.17, 0.12]	0.74	0%	0.70

SMD: standardised mean difference; I²: heterogeneity index; N_Cases: Number of patients with a failed AVF; N_Controls: Number of patients with a patent AVF; CRP: C-reactive protein; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol; PTH: Parathyroid hormone; TC: Total cholesterol; TG: Triglycerides. P-values in bold are significant.

*Calculated according to inverse-variance random-effects model

doi:10.1371/journal.pone.0159963.t004

13, 15, 16, 19, 20] or AVF thrombosis (Table 1) [11, 12, 18]. The study by Kaygin *et al*, was not clear in its definition of AVF failure [14], and was therefore excluded from these analyses. In line with the inclusion criteria for meta-analysis (assessed in at least 3 studies and ≥100 participants), albumin and CRP were the only 2 biomarkers which could be assessed in these sub-analyses (Fig 2). No significant association of either of these markers with AVF stenosis or thrombosis was observed. It was noted that the cases reported by Bojakowski *et al.* included a mixture of patients with failed AV due to either thrombosis or stenosis [10]. To eliminate potential confounding from this mixed population, meta-analyses assessing the association of albumin and CRP with AVF stenosis, and CRP with AVF thrombosis were repeated, excluding data from Bojakowski *et al.* [10]. No association of these markers with either AVF outcome was observed (Table 5). We were unable to assess the association of albumin with AVF thrombosis as only 2 studies remained after excluding data from Bojakowski and colleagues which violated inclusion criteria for the current study. A leave-one-out sensitivity analysis was also performed for each biomarker (S5 File).
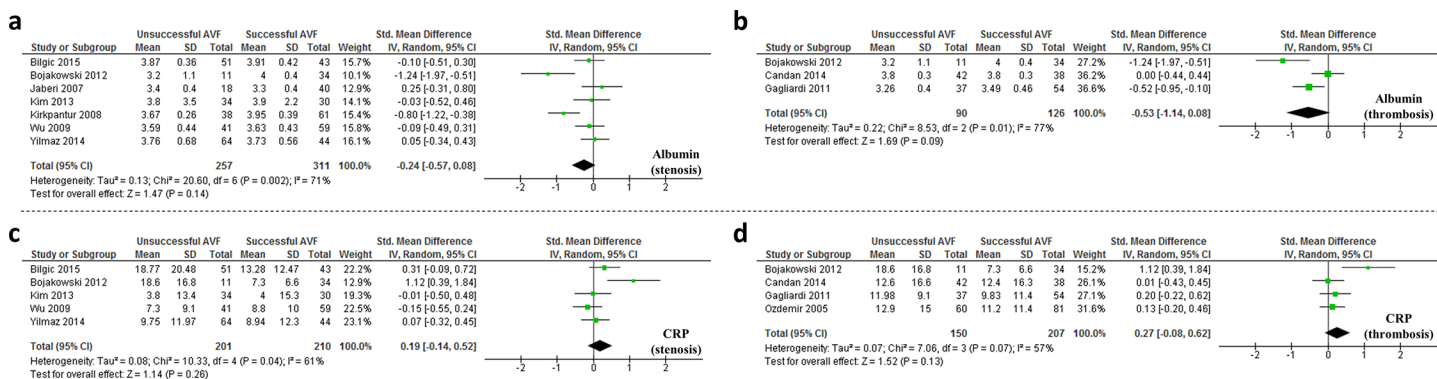
Fig 2. Circulating levels of albumin or CRP are not significantly associated with either AVF stenosis or AVF thrombosis in HD patients. Forest plot of meta-analysis data showing the association between circulating albumin with AVF (a) stenosis or (b) thrombosis; and circulating CRP with AVF (c) stenosis or (d) thrombosis.

doi:10.1371/journal.pone.0159963.g002

Table 5. Meta-analysis of the association of albumin and CRP with AVF stenosis and thrombosis.

AVF Outcome	Biomarker	Studies	N_{Cases}	$N_{Controls}$	SMD (95%CI)	*p* value*	I^2	I^2 *p*-value
Stenosis	Albumin	7	257	311	-0.24 [-0.57, 0.08]	0.14	71%	**0.002**
	Albumin†	6	246	277	-0.14 [-0.42, 0.15]	0.34	60%	**0.03**
	CRP	5	201	210	0.19 [-0.14, 0.52]	0.26	61%	**0.04**
	CRP†	4	190	176	0.06 [-0.15, 0.26]	0.59	0%	0.44
Thrombosis	Albumin	3	90	126	-0.53 [-1.14, 0.08]	0.09	77%	**0.01**
	CRP	4	150	207	0.27 [-0.08, 0.62]	0.13	57%	0.07
	CRP†	3	139	173	0.12 [-0.10, 0.34]	0.30	0%	0.83

SMD: standardised mean difference; I^2: heterogeneity index; CRP: C-reactive protein; N_{Cases}: Number of patients with a failed AVF; $N_{Controls}$: Number of patients with a patent AVF

*Calculated according to inverse-variance random-effects model

†Analysis excluded mixed population data from Bojakowski *et al*. P-values in bold are significant.

doi:10.1371/journal.pone.0159963.t005

Discussion

Main Findings

AVF failure is a significant cause of morbidity and expense in the HD population [2]. Older age, female sex, diabetes, and smaller vein calibre are established risk factors for AVF failure [22]. No comprehensive assessment of the association between circulating biochemical factors and AVF failure has been previously published. We analysed data from thirteen studies and included twelve biomarkers in a meta-analysis from a possible group of 48 [8–20]. There was no significant association between any of these 12 biomarkers with AVF failure due to any cause, or when AVF failure was specifically due to stenosis or thrombosis. A possible reason for this result is study heterogeneity, evidenced by high I^2 statistic and significant I^2 p-value in many analyses.

Sources of Heterogeneity

The studies included in this review varied in many aspects. One such variation is differences between the methods used to measure biomarker concentrations. Most studies failed to provide comprehensive details on laboratory methods and the medium in which biomarkers were quantified (e.g. plasma or serum), and for those that did provide this information, blood medium varied considerably between studies. Differences in methodology have also previously been acknowledged to generate heterogeneity of biomarker concentrations in other studies [19, 23]. Another source of heterogeneity is the timing in which blood samples were collected in relation to AVF assessment. Of the eleven studies included in the meta-analyses, only three clearly stated that blood collection occurred at the time of AVF failure [9, 11, 19]. The remainder took a single measurement at AVF creation [10, 14], a single measurement while the AVF was functional [18, 20], or collected several blood samples to generate a mean over time [12, 13, 15, 16]. These methods could potentially either precede biochemical changes associated with AVF failure or combine circulating biochemical parameters associated with patent AVFs with those of failing AVFs, thereby dampening any possible association. Thus few of the studies were appropriately designed to identify circulating markers of AVF failure.

The primary outcome assessed also varied amongst the included studies. For example, Gagliardi *et al.* investigated factors that influenced AVF failure due to thrombosis, whereas Jaberi *et al* investigated the factors influencing cephalic arch stenosis [12, 13]. Whilst both studies presented results in a way that allowed an association to be drawn between a biomarker and

AVF outcome, in reality the two populations themselves represent significantly different cohorts.

In an attempt to overcome limitations of inter-study heterogeneity, sub analyses were conducted to assess the association of biomarkers with AVF stenosis or AVF thrombosis specifically. Due to our meta-analysis inclusion criteria this analysis was limited to albumin and CRP. There was no statistically significant association found between either albumin or CRP with AVF stenosis or thrombosis. These sub-analyses are likely underpowered although heterogeneity appeared to be reduced since the I^2 statistics were lower than for analyses of all studies.

The location of the AVF differed between studies, although most investigations focussed on brachiocephalic or radiocephalic fistulae. Brachiocephalic fistulae are reported to have greater patency, although they are also associated with a greater incidence of complications, particularly steal syndrome [24–26]. Most studies provided little information on the follow-up time, length of time patients were on HD prior to the study, history of previous AVF events, prevalence of diabetes and medication usage, which are important determinants of AVF outcome [27]. Overall the identified studies failed to report important and well defined determinants of AVF outcome. The quality of clinical research performed in this area may be greatly improved by standardised definitions of parameters that should be included in such studies in order to guide future work.

Future Directions

Six potentially important biomarkers were not included in this study as they did not fulfil the specified inclusion criteria. Elevated fibrinogen has been reported to be significantly associated with AVF failure [14], although this is contradicted by another study where no association was found between fibrinogen and AVF failure [12]. Red blood cell distribution width (RDW), an indicator of anisocytosis, was reported to be significantly greater in patients with AVF failure in a single study [10]. Elevated RDW is also associated with other cardiovascular conditions such as coronary artery disease and myocardial infarction [28, 29], suggesting that increased RDW alone is unlikely to be specific to AVF failure. Plasma asymmetrical dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase, has also reported to be higher in patients with restenosis following a PTA, than patients whose AVF remained patent following the procedure [19]. Similarly, elevated levels of serum osteoprotegerin (OPG) have been reported to predict AVF stenosis [15], possibly linked to the histopathological similarities between AVF stenosis and atherosclerosis [12]. Soluble endothelial leukocyte adhesion molecule-1 (sE-selectin) has also been reported to be significantly elevated in patients with AVF stenosis [9]. E-selectin has been previously implicated in intimal hyperplasia [9]. Finally, significantly lower levels of the angiogenic cytokine vascular endothelial growth factor A (VEGF-A) were reported in patients with AVF thrombosis [11]. VEGF-A has been shown in animal models to have an anti-thrombotic effect and therefore may be a valuable prognostic tool [11]. Additional studies will be needed to conclusively establish the relationship between AVF functionality and these six biomarkers, however these data provide new directions for pathophysiological investigations into the failing AVF.

Study Limitations

This review had a number of limitations. Firstly, few data have been published in this field and thus sample sizes in our meta-analyses were small, reducing our analytical power. We set our eligibility for meta-analysis at biomarkers assessed in ≥3 studies with a total population of ≥100 participants, which we felt was a minimum requirement for such an analysis. As such we have excluded some biomarkers from the meta-analysis. Consequently, this also limited our ability to perform sub-analyses with regards to a specific cause of AVF failure (e.g. AVF

stenosis or thrombosis) for all biomarkers assessed in this meta-analysis. It is plausible that analysing a composite outcome (all AVF failure) may mask the effect of biomarkers on a specific cause of AVF failure. Secondly, there was significant heterogeneity between the studies, impacting on the strength of findings in the meta-analyses. Thirdly, AVF is just one form of VA and as such we have excluded a large proportion of studies that investigated the outcome of arteriovenous grafts and other forms of VA. However, an AVF is generally recognised as the superior form of VA and this was a motivating factor in our study design. Fourthly, we obtained data from publically available literature and therefore did not have access to primary data. In instances where data were unavailable, authors were contacted to obtain relevant data. We cannot exclude the potential influence of publication bias on our findings. Finally, we were limited to searching for articles published in the English language, and it is therefore possible that potentially useful papers detailing other markers of AVF failure in non-English journals were not included here. We acknowledge this as a potential source of bias in the findings of our analysis.

Conclusion

To our knowledge this meta-analysis represents the first comprehensive investigation of biomarkers associated with AVF failure. Our results demonstrate no conclusive association of any previously assessed biomarker with AVF failure, although it is important to note that the range of evaluated biomarkers is narrow and predominantly restricted to markers assessed in routine clinical investigations. We conclude that rigorously designed studies of biologically plausible biomarkers are needed to decide the clinical value of biomarkers for monitoring HD. Care must be taken during experimental design, to ensure study protocol effectively addresses the primary research question. For example, in investigations designed to correlate biomarkers with AVF failure, blood samples must be taken appropriate to the time of failure.

Supporting Information

S1 Fig. None of the 12 biomarkers were found to be significantly associated with AVF failure. Forest plot of meta-analysis data showing the lack of an association between AVF failure and circulating (a) albumin; (b) calcium; (c) creatinine; (d) C-reactive protein (CRP); (e) ferritin; (f) haemoglobin; (g) high density lipoprotein cholesterol (HDL-C); (h) low density lipoprotein cholesterol (LDL-C); (i) parathyroid hormone (PTH); (j) phosphorus; (k) total cholesterol (TC); and triglycerides.
(PDF)

S2 Fig. Circulating levels of albumin or CRP were not significantly associated with AVF stenosis or AVF thrombosis in HD patients, even when data from a mixed population (Bojakowski *et al.*) were removed. Forest plot of meta-analysis data showing the lack of an association between circulating albumin with AVF (a) stenosis; and circulating CRP with AVF (b) stenosis or (c) thrombosis, when data from Bojakowski *et al.* is removed.
(PDF)

S1 File. Search strategies.
(PDF)

S2 File. Data extraction form.
(PDF)

S3 File. Results of the modified Ottawa-Newcastle tool to assess the risk of bias in cohort studies.
(PDF)

S4 File. Results of the modified Ottawa-Newcastle tool to assess the risk of bias in case control studies.
(PDF)

S5 File. Leave-one-out sensitivity tests for all meta-analyses
(PDF)

S1 Table. Blood collection and laboratory methods used to quantify biomarkers. PTH: parathyroid hormone; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; CRP: C-reactive protein; sE-Selectin: soluble E-selectin; eEPCR: soluble endothelial protein C receptor; TG: triglycerides; WBC: white blood cell count; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; RDW: red blood cell distribution width; RBC: red blood cell count; Ca x P: calcium times phosphate; VEGF-A: vascular endothelial growth factor A; MIS: malnutrition inflammation score; CMV: cytomegalovirus; OPG: osteoprotegerin; TC: total cholesterol; ADMA: asymmetrical dimethylarginine; NLR: neutrophil-lymphocyte ratio.
(DOCX)

Author Contributions

Conceived and designed the experiments: AJR DRM JVM JG. Analyzed the data: SKM. Wrote the paper: SKM. Literature search: SKM AJR. Data extraction: SKM AJR DRM APB. Reviewed final draft: SKM AJR DRM APB JVM JG.

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S1 Fig. None of the 12 biomarkers were found to be significantly associated with AVF failure.

Forest plot of meta-analysis data showing the lack of an association between AVF failure and circulating (a) albumin; (b) calcium; (c) creatinine; (d) C-reactive protein (CRP); (e) ferritin; (f) haemoglobin; (g) high density lipoprotein cholesterol (HDL-C); (h) low density lipoprotein cholesterol (LDL-C); (i) parathyroid hormone (PTH); (j) phosphorus; (k) total cholesterol (TC); and triglycerides.

S2 Fig. Circulating levels of albumin or CRP were not significantly associated with AVF stenosis or AVF thrombosis in HD patients, even when data from a mixed population (Bojakowski *et al*.) were removed.

Forest plot of meta-analysis data showing the lack of an association between circulating albumin with AVF (a) stenosis; and circulating CRP with AVF (b) stenosis or (c) thrombosis, when data from Bojakowski *et al*. is removed.

S1 File. Search strategies.

S2 File. Results of the modified Ottawa-Newcastle tool to assess the risk of bias in cohort studies.

S3 File. Results of the modified Ottawa-Newcastle tool to assess the risk of bias in case control studies.

S4 File. Leave-one-out sensitivity tests for all meta-analyses

S1 Table. Blood collection and laboratory methods used to quantify biomarkers.

a Albumin

Study or Subgroup	Unsuccessful AVF Mean	SD	Total	Successful AVF Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Bilgic 2015	3.87	0.36	51	3.91	0.42	43	10.2%	-0.10 [-0.51, 0.30]
Bojakowski 2012	3.2	1.1	11	4	0.4	34	8.9%	-1.24 [-1.97, -0.51]
Candan 2014	3.8	0.3	42	3.8	0.3	38	10.1%	0.00 [-0.44, 0.44]
Gagliardi 2011	3.26	0.4	37	3.49	0.46	54	10.1%	-0.52 [-0.95, -0.10]
Jaberi 2007	3.4	0.4	18	3.3	0.4	40	9.6%	0.25 [-0.31, 0.80]
Kaygin 2013	3	0.8	75	3.96	0.4	311	10.5%	-1.91 [-2.19, -1.62]
Kim 2013	3.8	3.5	34	3.9	2.2	30	9.9%	-0.03 [-0.52, 0.46]
Kirkpantur 2008	3.67	0.26	38	3.95	0.39	61	10.1%	-0.80 [-1.22, -0.38]
Wu 2009	3.59	0.44	41	3.63	0.43	59	10.2%	-0.09 [-0.49, 0.31]
Yilmaz 2014	3.76	0.68	64	3.73	0.56	44	10.3%	0.05 [-0.34, 0.43]
Total (95% CI)			411			714	100.0%	-0.44 [-0.95, 0.07]

Heterogeneity: Tau² = 0.62; Chi² = 128.09, df = 9 (P < 0.00001); I² = 93%
Test for overall effect: Z = 1.69 (P = 0.09)

b Calcium

Study or Subgroup	Unsuccessful AVF Mean	SD	Total	Successful AVF Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Bilgic 2015	8.4	0.52	51	8.5	0.65	43	22.8%	-0.17 [-0.58, 0.24]
Jaberi 2007	9.16	0.6	18	9.08	0.72	40	12.2%	0.12 [-0.44, 0.67]
Kim 2013	8.5	4.1	34	8.6	2.7	30	15.7%	-0.03 [-0.52, 0.46]
Wu 2009	9.97	1.04	41	9.89	0.86	59	23.8%	0.08 [-0.31, 0.48]
Yilmaz 2014	8.02	0.64	64	8.1	0.55	44	25.6%	-0.13 [-0.52, 0.25]
Total (95% CI)			208			216	100.0%	-0.04 [-0.24, 0.15]

Heterogeneity: Tau² = 0.00; Chi² = 1.29, df = 4 (P = 0.86); I² = 0%
Test for overall effect: Z = 0.43 (P = 0.67)

c Creatinine

Study or Subgroup	Unsuccessful AVF Mean	SD	Total	Successful AVF Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Bojakowski 2012	5.2	1.9	11	5.1	1.9	34	9.0%	0.05 [-0.63, 0.73]
Kaygin 2013	4.2	2.6	75	4.1	2.5	311	65.1%	0.04 [-0.21, 0.29]
Wu 2009	10.6	2.2	41	10.2	2.2	59	26.0%	0.18 [-0.22, 0.58]
Total (95% CI)			127			404	100.0%	0.08 [-0.13, 0.28]

Heterogeneity: Tau² = 0.00; Chi² = 0.35, df = 2 (P = 0.84); I² = 0%
Test for overall effect: Z = 0.74 (P = 0.46)

d CRP

Study or Subgroup	Unsuccessful AVF Mean	SD	Total	Successful AVF Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Bilgic 2015	18.77	20.48	51	13.28	12.47	43	11.2%	0.31 [-0.09, 0.72]
Bojakowski 2012	18.6	16.8	11	7.3	6.6	34	10.8%	1.12 [0.39, 1.84]
Candan 2014	12.6	16.6	42	12.4	16.3	38	11.1%	0.01 [-0.43, 0.45]
Gagliardi 2011	11.98	9.1	37	9.83	11.4	54	11.1%	0.20 [-0.22, 0.62]
Kaygin 2013	18.6	4.3	75	4.6	2.2	311	11.1%	5.11 [4.67, 5.55]
Kim 2013	3.8	13.4	34	4	15.3	30	11.1%	-0.01 [-0.50, 0.48]
Ozdemir 2005	12.9	15	60	11.2	11.4	81	11.2%	0.13 [-0.20, 0.46]
Wu 2009	7.3	9.1	41	8.8	10	59	11.1%	-0.15 [-0.55, 0.24]
Yilmaz 2014	9.75	11.97	64	8.94	12.3	44	11.2%	0.07 [-0.32, 0.45]
Total (95% CI)			415			694	100.0%	0.75 [-0.32, 1.82]

Heterogeneity: Tau² = 2.62; Chi² = 449.81, df = 8 (P < 0.00001); I² = 98%
Test for overall effect: Z = 1.38 (P = 0.17)

e Ferritin

Study or Subgroup	Unsuccessful AVF Mean	SD	Total	Successful AVF Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Bilgic 2015	422.4	240.8	51	439.1	230.7	43	20.8%	-0.07 [-0.48, 0.34]
Bojakowski 2012	170.4	104.7	11	235.7	314.5	34	7.4%	-0.23 [-0.91, 0.45]
Candan 2014	855.1	714.9	42	890.6	619.1	38	17.8%	-0.05 [-0.49, 0.39]
Ozdemir 2005	552.4	821.6	60	497.6	308.4	81	30.7%	0.09 [-0.24, 0.43]
Yilmaz 2014	542.43	230.45	64	539.15	286.37	44	23.3%	0.01 [-0.37, 0.40]
Total (95% CI)			228			240	100.0%	-0.01 [-0.19, 0.18]

Heterogeneity: Tau² = 0.00; Chi² = 0.90, df = 4 (P = 0.92); I² = 0%
Test for overall effect: Z = 0.10 (P = 0.92)

f Haemoglobin

Study or Subgroup	Unsuccessful AVF Mean	SD	Total	Successful AVF Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Bilgic 2015	10.85	1.15	51	10.9	1.26	43	19.7%	-0.04 [-0.45, 0.36]
Bojakowski 2012	9.7	1	11	10.9	1.5	34	9.0%	-0.84 [-1.55, -0.14]
Candan 2014	11.6	1.5	42	11.3	1.3	38	17.8%	0.21 [-0.23, 0.65]
Jaberi 2007	11.6	1.5	18	11.6	1.3	40	13.0%	0.00 [-0.56, 0.56]
Kirkpantur 2008	10.9	1	38	11.2	1	61	19.6%	-0.30 [-0.70, 0.11]
Yilmaz 2014	10.83	1.97	64	10.75	1.82	44	20.9%	0.04 [-0.34, 0.43]
Total (95% CI)			224			260	100.0%	-0.10 [-0.33, 0.14]

Heterogeneity: Tau² = 0.03; Chi² = 7.78, df = 5 (P = 0.17); I² = 36%
Test for overall effect: Z = 0.80 (P = 0.42)

g HDL-C

Study or Subgroup	Unsuccessful AVF Mean	SD	Total	Successful AVF Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Bilgic 2015	39.6	10.3	51	38.7	11.2	43	14.5%	0.08 [-0.32, 0.49]
Bojakowski 2012	52.1	18.5	11	56.7	17.6	34	13.2%	-0.25 [-0.94, 0.43]
Candan 2014	33.9	13	42	32.4	8.9	38	14.4%	0.13 [-0.31, 0.57]
Kaygin 2013	42.8	12.5	75	39.6	11.8	311	15.0%	0.27 [0.01, 0.52]
Kirkpantur 2008	31.4	4.4	38	44	7	61	14.1%	-2.04 [-2.53, -1.54]
Wu 2009	54	19	41	50	17	59	14.5%	0.22 [-0.18, 0.62]
Yilmaz 2014	31.8	12.6	64	51.5	11.9	44	14.4%	-1.59 [-2.03, -1.15]
Total (95% CI)			322			590	100.0%	-0.45 [-1.12, 0.23]

Heterogeneity: Tau² = 0.77; Chi² = 111.79, df = 6 (P < 0.00001); I² = 95%
Test for overall effect: Z = 1.30 (P = 0.20)

h LDL-C

Study or Subgroup	Unsuccessful AVF Mean	SD	Total	Successful AVF Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Bilgic 2015	154.5	32.6	51	128.7	28.6	43	14.4%	0.83 [0.41, 1.25]
Bojakowski 2012	108.6	48.1	11	99.5	45.7	34	12.9%	0.19 [-0.49, 0.87]
Candan 2014	98	35.1	42	95.9	33	38	14.3%	0.06 [-0.38, 0.50]
Kaygin 2013	118.7	28.6	75	114.8	28.3	311	15.2%	0.14 [-0.12, 0.39]
Kirkpantur 2008	62.8	11	38	97.4	19	61	14.0%	-2.09 [-2.59, -1.59]
Wu 2009	113	33	41	102	30	59	14.5%	0.35 [-0.05, 0.75]
Yilmaz 2014	102.69	36.13	64	99.86	39.49	44	14.6%	0.07 [-0.31, 0.46]
Total (95% CI)			322			590	100.0%	-0.06 [-0.64, 0.53]

Heterogeneity: Tau² = 0.57; Chi² = 85.58, df = 6 (P < 0.00001); I² = 93%
Test for overall effect: Z = 0.19 (P = 0.85)

i PTH

Study or Subgroup	Unsuccessful AVF Mean	SD	Total	Successful AVF Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Bilgic 2015	332.8	160.5	51	319.5	204.3	43	22.5%	0.07 [-0.33, 0.48]
Candan 2014	267.5	229.5	42	311.4	316.1	38	19.2%	-0.16 [-0.60, 0.28]
Ozdemir 2005	449.4	363.4	60	492	409.9	81	33.2%	-0.11 [-0.44, 0.23]
Yilmaz 2014	371.7	301.04	64	361.57	327.48	44	25.1%	0.03 [-0.35, 0.42]
Total (95% CI)			217			206	100.0%	-0.04 [-0.23, 0.15]

Heterogeneity: Tau² = 0.00; Chi² = 0.87, df = 3 (P = 0.83); I² = 0%
Test for overall effect: Z = 0.43 (P = 0.67)

j Phosphorus

Study or Subgroup	Unsuccessful AVF Mean	SD	Total	Successful AVF Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Bilgic 2015	5.8	1.9	51	5.75	1.79	43	22.9%	0.03 [-0.38, 0.43]
Jaberi 2007	6.25	2.2	18	5.14	1.46	40	11.9%	0.64 [0.07, 1.21]
Kim 2013	5.3	26.2	34	5.2	26.8	30	15.9%	0.00 [-0.49, 0.49]
Wu 2009	4.39	1.36	41	4.45	1.74	59	23.8%	-0.04 [-0.44, 0.36]
Yilmaz 2014	6.29	1.51	64	6.13	1.49	44	25.5%	0.11 [-0.28, 0.49]
Total (95% CI)			208			216	100.0%	0.10 [-0.10, 0.30]

Heterogeneity: Tau² = 0.00; Chi² = 4.15, df = 4 (P = 0.39); I² = 4%
Test for overall effect: Z = 0.99 (P = 0.32)

k TC

Study or Subgroup	Unsuccessful AVF Mean	SD	Total	Successful AVF Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Candan 2014	167	45.3	42	160.7	43.7	38	20.9%	0.14 [-0.30, 0.58]
Gagliardi 2011	148.24	23.5	37	136	35.6	54	21.9%	0.39 [-0.03, 0.81]
Kaygin 2013	183.5	28.2	75	172.3	44.9	311	34.3%	0.26 [0.01, 0.52]
Kirkpantur 2008	145.6	35.1	38	155	34.5	61	22.8%	-0.27 [-0.68, 0.14]
Total (95% CI)			192			464	100.0%	0.14 [-0.12, 0.41]

Heterogeneity: Tau² = 0.04; Chi² = 6.05, df = 3 (P = 0.11); I² = 50%
Test for overall effect: Z = 1.07 (P = 0.28)

l Triglycerides

Study or Subgroup	Unsuccessful Mean	SD	Total	Successful Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Bilgic 2015	279.5	90.4	51	288.9	106.6	43	12.6%	-0.10 [-0.50, 0.31]
Bojakowski 2012	158.4	75.8	11	148.6	82.4	34	4.5%	0.12 [-0.56, 0.80]
Candan 2014	181.8	84.7	42	170.1	84.7	38	10.7%	0.14 [-0.30, 0.58]
Kaygin 2013	148.6	74.8	75	153.8	82.2	311	32.6%	-0.06 [-0.32, 0.19]
Kirkpantur 2008	143.6	66.2	38	161.3	61.5	61	12.5%	-0.28 [-0.68, 0.13]
Wu 2009	175	105	41	155	80	59	13.0%	0.22 [-0.18, 0.62]
Yilmaz 2014	195.58	89.41	64	198.78	96.436	44	14.1%	-0.03 [-0.42, 0.35]
Total (95% CI)			322			590	100.0%	-0.02 [-0.17, 0.12]

Heterogeneity: Tau² = 0.00; Chi² = 3.80, df = 6 (P = 0.70); I² = 0%
Test for overall effect: Z = 0.33 (P = 0.74)

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Study or Subgroup	Unsuccessful AVF			Successful AVF			Weight	Std. Mean Difference IV, Random, 95% CI	Std. Mean Difference IV, Random, 95% CI
	Mean	SD	Total	Mean	SD	Total			
Bilgic 2015	3.87	0.36	51	3.91	0.42	43	17.7%	-0.10 [-0.51, 0.30]	
Bojakowski 2012	3.2	1.1	11	4	0.4	34	0.0%	-1.24 [-1.97, -0.51]	
Jaberi 2007	3.4	0.4	18	3.3	0.4	40	13.4%	0.25 [-0.31, 0.80]	
Kim 2013	3.8	3.5	34	3.9	2.2	30	15.2%	-0.03 [-0.52, 0.46]	
Kirkpantur 2008	3.67	0.26	38	3.95	0.39	61	17.3%	-0.80 [-1.22, -0.38]	
Wu 2009	3.59	0.44	41	3.63	0.43	59	18.0%	-0.09 [-0.49, 0.31]	
Yilmaz 2014	3.76	0.68	64	3.73	0.56	44	18.5%	0.05 [-0.34, 0.43]	
Total (95% CI)			246			277	100.0%	-0.14 [-0.42, 0.15]	

Albumin (Stenosis)

Heterogeneity: Tau² = 0.07; Chi² = 12.54, df = 5 (P = 0.03); I² = 60%
Test for overall effect: Z = 0.95 (P = 0.34)

Study or Subgroup	Unsuccessful AVF			Successful AVF			Weight	Std. Mean Difference IV, Random, 95% CI	Std. Mean Difference IV, Random, 95% CI
	Mean	SD	Total	Mean	SD	Total			
Bilgic 2015	18.77	20.48	51	13.28	12.47	43	25.8%	0.31 [-0.09, 0.72]	
Bojakowski 2012	18.6	16.8	11	7.3	6.6	34	0.0%	1.12 [0.39, 1.84]	
Kim 2013	3.8	13.4	34	4	15.3	30	17.9%	-0.01 [-0.50, 0.48]	
Wu 2009	7.3	9.1	41	8.8	10	59	27.1%	-0.15 [-0.55, 0.24]	
Yilmaz 2014	9.75	11.97	64	8.94	12.3	44	29.2%	0.07 [-0.32, 0.45]	
Total (95% CI)			190			176	100.0%	0.06 [-0.15, 0.26]	

CRP (Stenosis)

Heterogeneity: Tau² = 0.00; Chi² = 2.69, df = 3 (P = 0.44); I² = 0%
Test for overall effect: Z = 0.53 (P = 0.59)

Study or Subgroup	Unsuccessful AVF			Successful AVF			Weight	Std. Mean Difference IV, Random, 95% CI	Std. Mean Difference IV, Random, 95% CI
	Mean	SD	Total	Mean	SD	Total			
Bojakowski 2012	18.6	16.8	11	7.3	6.6	34	0.0%	1.12 [0.39, 1.84]	
Candan 2014	12.6	16.6	42	12.4	16.3	38	26.2%	0.01 [-0.43, 0.45]	
Gagliardi 2011	11.98	9.1	37	9.83	11.4	54	28.7%	0.20 [-0.22, 0.62]	
Ozdemir 2005	12.9	15	60	11.2	11.4	81	45.1%	0.13 [-0.20, 0.46]	
Total (95% CI)			139			173	100.0%	0.12 [-0.10, 0.34]	

CRP (Thrombosis)

Heterogeneity: Tau² = 0.00; Chi² = 0.38, df = 2 (P = 0.83); I² = 0%
Test for overall effect: Z = 1.04 (P = 0.30)

S1 File. Search Strategies.

All fields:	"AVF" AND "Vascular Access"
Title/Abstract: *One term per search*	"biomarker" or "concentration" or "function" or "dysfunction" or "maturation" or "patency" or "failure" or "survival" or "thrombo*" or "steno*" or "factor" or "predict*" or "serum" or "plasma" or "circulating" or "risk factor" or "blood"

<u>MEDLINE</u>
Date limiter: January 1966 – December 2015
Date of Search: 22nd December 2015
Total Number of hits: 2266

<u>EMBASE</u>
Date limiter: January 1966 – December 2015
Date of Search: 22nd December 2015
Total Number of hits: 1817

<u>COCHRANE LIBRARY</u>
Date limiter: January 1966 – December 2015
Date of Search: 22nd December 2015
Total Number of hits: 152

S3 File. Results of the modified Ottawa-Newcastle tool to assess risk of bias in cohort studies

Question	Reference								
	Baumann	**Bojakowski**	**Gagliardi**	**Jaberi**	**Kaygin**	**Kirkpantur**	**Masaki**	**Wu**	**Yilmaz**
1. Was selection of exposed and non-exposed cohorts drawn from the same population?	Definitely Yes	Probably Yes	Probably Yes	Probably Yes	Probably Yes	Definitely Yes	Probably Yes	Probably No	Probably No
2. Can we be confident in the assessment of exposure?	Definitely Yes	Definitely Yes	Definitely Yes	Probably Yes	Probably Yes	Probably Yes	Probably Yes	Probably No	Probably Yes
3. Can we be confident that the outcome of interest was not present at start of study?	Definitely Yes	Definitely Yes	Definitely Yes	Definitely No	Definitely Yes	Probably Yes	Probably Yes	Definitely No	Probably Yes
4. Did the study match exposed and unexposed for all variables that are associated with the outcome of interest, or did the statistical analysis adjust for these prognostic variables?	Mostly Yes	Mostly Yes	Mostly Yes	Mostly No	Mostly Yes	Mostly Yes	Mostly Yes	Mostly Yes	Mostly Yes
5. Can we be confident in the assessment of the presence or absence of prognostic factors?	Probably Yes	Definitely Yes	Definitely Yes	Probably Yes	Definitely Yes	Probably Yes	Definitely Yes	Probably Yes	Probably Yes
6. Can we be confident in the assessment of outcome?	Definitely Yes	Definitely Yes	Probably Yes	Probably Yes	Probably Yes	Definitely Yes	Definitely Yes	Definitely Yes	Definitely Yes
7. Was the follow up of cohorts adequate?	Definitely Yes	Definitely Yes	Probably Yes	Probably Yes	Definitely Yes	Probably Yes	Probably Yes	Probably Yes	Definitely Yes
8. Were co-interventions similar between groups?	Probably Yes	Definitely Yes	Probably Yes	Probably Yes	Probably Yes	Probably Yes	Probably Yes	Probably Yes	Probably Yes
Summary of overall risk of bias	Low	Very Low	Low	High	Low	Medium	Medium	High	High

NB: The risk of bias was considered very low if 100% questions were answered with either a 'Definitely Yes' or 'Probably/Mostly Yes', with at least 6/8 being 'Definitely Yes'; low if 100% questions were answered with either a 'Definitely Yes' or 'Probably/Mostly Yes', with at least 3/8 being 'Definitely Yes'; medium if 100% questions were answered with either a 'Definitely Yes' or 'Probably/Mostly Yes', with 2/8 or less being 'Definitely Yes'; High if 3/8 or less questions answered with either 'Definitely No' or 'Probably/Mostly No'; Very high if 4/8 or more questions were answered with either 'Definitely No' or 'Probably/Mostly No.'

Definitely Yes: Very low risk of bias; Probably/Mostly yes: Low risk of bias; Probably/Mostly no: Medium risk of bias; Definitely No: high risk of bias. Modified Ottawa-Newcastle Tool (https://distillercer.com/resources/)

S4 File. Results of the modified Ottawa-Newcastle tool to assess risk of bias in case control studies

Question	Reference			
	Bilgic	**Candan**	**Kim**	**Ozdemir**
1. Can we be confident in the assessment of exposure?	Probably Yes	Definitely Yes	Definitely Yes	Probably Yes
2. Can we be confident that cases had developed the outcome of interest and controls had not?	Definitely Yes	Probably Yes	Definitely Yes	Probably Yes
3. Were the cases (those who were exposed and developed the outcome of interest) properly selected?	Definitely Yes	Definitely Yes	Probably Yes	Probably Yes
4. Were the controls (those who were exposed and did not develop the outcome of interest) properly selected?	Definitely Yes	Definitely Yes	Probably Yes	Probably Yes
5. Were cases and controls matches according to important prognostic variables or was statistical adjustment carried out for those variables?	Probably Yes	Probably Yes	Probably Yes	Probably Yes
Summary of overall risk of bias	Low	Low	Medium	Medium

NB: The risk of bias was considered very low if 100% of questions were answered with 'Definitely Yes'; low if 100% of questions were answered with either a 'Definitely Yes' or 'Probably/Mostly Yes', with at least 3/5 being 'Definitely Yes'; Medium if 100% of questions were answered with either a 'Definitely Yes' or 'Probably/Mostly Yes', with at least 2/5 or less being 'Definitely Yes'; High if 2/5 or less questions answered with either 'Definitely No' or 'Probably/Mostly No'; Very high if 3/5 or more questions answered with either 'Definitely No' or 'Probably/Mostly No.'
Definitely Yes: Very low risk of bias; Probably/Mostly yes: Low risk of bias; Probably/Mostly no: Medium risk of bias; Definitely No: high risk of bias.
Modified Ottawa-Newcastle Tool (https://distillercer.com/resources/)

S5 File. Leave-one-out sensitivity analyses for all meta-analyses

Leave-one-out sensitivity test for the meta-analysis assessing Albumin and all AVF failure

Study Excluded	No. Studies	N_{Cases}	$N_{Controls}$	SMD (95%CI)	*p* value*	I^2	I^2 *p* value
Nil	10	411	714	-0.44 [-0.95, 0.07]	0.09	93%	**< 0.001**
Bilgic	9	360	671	-0.48 [-1.04, 0.08]	0.09	93%	**< 0.001**
Bojakowski	9	400	680	-0.36 [-0.90, 0.18]	0.19	94%	**< 0.001**
Candan	9	369	676	-0.49 [-1.04, 0.06]	0.08	93%	**< 0.001**
Gagliardi	9	374	660	-0.43 [-1.00, 0.14]	0.14	94%	**< 0.001**
Jaberi	9	393	674	-0.51 [-1.05, 0.02]	0.06	93%	**< 0.001**
Kaygin	9	336	403	-0.25 [-0.51, 0.02]	0.07	66%	**<0.003**
Kim	9	377	684	-0.48 [-1.03, 0.07]	0.08	93%	**< 0.001**
Kirkpantur	9	373	653	-0.04 [-0.97, 0.17]	0.17	94%	**< 0.001**
Wu	9	370	655	-0.48 [-1.04, 0.08]	0.09	93%	**< 0.001**
Yilmaz	9	347	670	-0.50 [-1.05, 0.05]	0.08	93%	**< 0.001**

SMD: standardised mean difference; I^2: heterogeneity index; *Calculated according to inverse-variance random-effects model. P-values in bold considered significant.

Leave-one-out sensitivity test for the meta-analysis assessing Calcium and all AVF failure

Study Excluded	No. Studies	N_{Cases}	$N_{Controls}$	SMD (95%CI)	*p* value*	I^2	I^2 *p* value
Nil	5	208	216	-0.04 [-0.24, 0.15]	0.67	0%	0.86
Bilgic	4	157	173	-0.00 [-0.23, 0.22]	0.96	0%	0.85
Jaberi	4	190	176	-0.06 [-0.27, 0.14]	0.54	0%	0.82
Kim	4	174	186	-0.05 [-0.26, 0.17]	0.67	0%	0.73
Wu	4	167	157	-0.08 [-0.30, 0.14]	0.47	0%	0.86
Yilmaz	4	144	172	-0.01 [-0.24, 0.21]	0.91	0%	0.80

SMD: standardised mean difference; I^2: heterogeneity index; *Calculated according to inverse-variance random-effects model. P-values in bold considered significant.

Leave-one-out sensitivity test for the meta-analysis assessing Creatinine and all AVF failure

Study Excluded	No. Studies	N_{Cases}	$N_{Controls}$	SMD (95%CI)	*p* value*	I^2	I^2 *p* value
Nil	3	127	404	0.08 [-0.13, 0.28]	0.46	0%	0.84
Bojakowski	2	116	370	0.08 [-0.13, 0.29]	0.46	0%	0.56
Kaygin	2	52	93	0.15 [-0.20, 0.49]	0.40	0%	0.75
Wu	2	86	345	0.04 [-0.20, 0.28]	0.73	0%	0.97

SMD: standardised mean difference; I^2: heterogeneity index; *Calculated according to inverse-variance random-effects model. P-values in bold considered significant.

Leave-one-out sensitivity test for the meta-analysis assessing CRP and all AVF failure

Study Excluded	No. Studies	N_{Cases}	$N_{Controls}$	SMD (95%CI)	*p* value*	I^2	I^2 *p* value
Nil	9	415	694	0.75 [-0.32, 1.82]	0.17	98%	**< 0.001**
Bilgic	8	364	651	0.81 [-0.41, 2.02]	0.19	98%	**< 0.001**
Bojakowski	8	404	660	0.71 [-0.45, 1.86]	0.23	98%	**< 0.001**
Candan	8	373	656	0.84 [-0.35, 2.04]	0.17	98%	**< 0.001**
Gagliardi	8	378	640	0.82 [-0.39, 2.03]	0.18	98%	**< 0.001**
Kaygin	8	340	383	0.14 [-0.05, 0.33]	0.14	35%	0.15
Kim	8	381	664	0.85 [-0.34, 2.03]	0.16	98%	**< 0.001**
Ozdemir	8	355	613	0.83 [-0.41, 2.07]	0.19	98%	**< 0.001**
Wu	8	374	635	0.87 [-0.33, 2.06]	0.16	98%	**< 0.001**
Yilmaz	8	351	650	0.84 [-0.38, 2.05]	0.18	98%	**< 0.001**

CRP: C-reactive protein; SMD: standardised mean difference; I^2: heterogeneity index; *Calculated according to inverse-variance random-effects model. P-values in bold considered significant.

Leave-one-out sensitivity test for the meta-analysis assessing Ferritin and all AVF failure

Study Excluded	No. Studies	N_{Cases}	$N_{Controls}$	SMD (95%CI)	*p* value*	I^2	I^2 *p* value
Nil	5	228	240	-0.01 [-0.19, 0.18]	0.92	0%	0.92
Bilgic	4	177	197	0.01 [-0.20, 0.21]	0.95	0%	0.85
Bojakowski	4	217	206	0.01 [-0.18, 0.20]	0.93	0%	0.93
Candan	4	186	202	0.00 [-0.20, 0.20]	1.00	0%	0.84
Ozdemir	4	168	159	-0.05 [-0.28, 0.17]	0.63	0%	0.95
Yilmaz	4	164	196	-0.02 [-0.23, 0.20]	0.88	0%	0.83

SMD: standardised mean difference; I^2: heterogeneity index; *Calculated according to inverse-variance random-effects model. P-values in bold considered significant.

Leave-one-out sensitivity test for the meta-analysis assessing Haemoglobin and all AVF failure

Study Excluded	No. Studies	N_{Cases}	$N_{Controls}$	SMD (95%CI)	*p* value*	I^2	I^2 *p* value
Nil	6	224	260	-0.10 [-0.33, 0.14]	0.42	36%	0.17
Bilgic	5	173	217	-0.12 [-0.42, 0.18]	0.43	48%	0.10
Bojakowski	5	213	226	-0.02 [-0.22, 0.17]	0.80	0%	0.57
Candan	5	182	222	-0.16 [-0.41, 0.09]	0.21	30%	0.22
Jaberi	5	206	220	-0.12 [-0.40, 0.16]	0.40	48%	0.10
Kirkpantur	5	224	260	-0.10 [-0.33, 0.14]	0.42	38%	0.17
Yilmaz	5	160	216	-0.14 [-0.43, 0.15]	0.35	45%	0.12

SMD: standardised mean difference; I^2: heterogeneity index; *Calculated according to inverse-variance random-effects model. P-values in bold considered significant.

Leave-one-out sensitivity test for the meta-analysis assessing HDL-C and all AVF failure

Study Excluded	No. Studies	N Cases	N Controls	SMD (95%CI)	p value*	I²	I² p value
Nil	7	322	590	-0.45 [-1.12, 0.23]	0.20	95%	< 0.001
Bilgic	6	271	547	-0.54 [-1.33, 0.26]	0.19	95%	< 0.001
Bojakowski	6	311	556	-0.48 [-1.23, 0.28]	0.22	96%	< 0.001
Candan	6	280	552	-0.54 [-1.33, 0.24]	0.17	95%	< 0.001
Kaygin	6	247	279	-0.57 [-1.37, 0.22]	0.16	64%	< 0.001
Kirkpantur	6	284	529	-0.18 [-0.73, 0.37]	0.52	91%	< 0.001
Wu	6	281	531	-0.56 [-1.35, 0.23]	0.16	95%	< 0.001
Yilmaz	6	258	546	-0.25 [-0.88, 0.38]	0.44	93%	< 0.001

HDL-C: high density lipoprotein cholesterol; SMD: standardised mean difference; I²: heterogeneity index; *Calculated according to inverse-variance random-effects model. P-values in bold considered significant.

Leave-one-out sensitivity test for the meta-analysis assessing LDL-C and all AVF failure

Study Excluded	No. Studies	N Cases	N Controls	SMD (95%CI)	p value*	I²	I² p value
Nil	7	322	590	-0.06 [-0.64, 0.53]	0.85	93%	< 0.001
Bilgic	6	271	547	-0.21 [-0.83, 0.42]	0.52	93%	< 0.001
Bojakowski	6	311	556	-0.09 [-0.74, 0.55]	0.77	94%	< 0.001
Candan	6	280	552	-0.08 [-0.76, 0.61]	0.82	94%	< 0.001
Kaygin	6	247	279	-0.09 [-0.86, 0.67]	0.81	94%	< 0.001
Kirkpantur	6	284	529	0.27 [0.04, 0.50]	**0.02**	49%	0.08
Wu	6	281	531	-0.13 [-0.81, 0.56]	0.72	94%	< 0.001
Yilmaz	6	258	546	-0.08 [-0.78, 0.62]	0.82	94%	< 0.001

LDL-C: low density lipoprotein cholesterol; SMD: standardised mean difference; I²: heterogeneity index; *Calculated according to inverse-variance random-effects model. P-values in bold considered significant.

Leave-one-out sensitivity test for the meta-analysis assessing PTH and all AVF failure

Study Excluded	No. Studies	N Cases	N Controls	SMD (95%CI)	p value*	I²	I² p value
Nil	4	217	206	-0.04 [-0.23, 0.15]	0.67	0%	0.83
Bilgic	3	166	163	-0.08 [-0.29, 0.14]	0.50	0%	0.79
Candan	3	175	168	-0.01 [-0.23, 0.20]	0.90	0%	0.76
Ozdemir	3	157	125	-0.01 [-0.24, 0.23]	0.94	0%	0.72
Yilmaz	3	153	162	-0.07 [-0.29, 0.16]	0.56	0%	0.71

PTH: parathyroid hormone; SMD: standardised mean difference; I²: heterogeneity index; *Calculated according to inverse-variance random-effects model. P-values in bold considered significant.

Leave-one-out sensitivity test for the meta-analysis assessing Phosphorus and all AVF failure

Study Excluded	No. Studies	N_{Cases}	$N_{Controls}$	SMD (95%CI)	p value*	I^2	I^2 p value
Nil	5	208	216	0.10 [-0.10, 0.30]	0.32	4%	0.39
Bilgic	4	157	173	0.13 [-0.13, 0.39]	0.32	25%	0.26
Jaberi	4	190	176	0.03 [-0.18, 0.24]	0.79	0%	0.97
Kim	4	174	186	0.13 [-0.12, 0.38]	0.31	25%	0.26
Wu	4	167	157	0.15 [-0.10, 0.39]	0.23	16%	0.31
Yilmaz	4	144	172	0.11 [-0.16, 0.38]	0.42	28%	0.25

SMD: standardised mean difference; I^2: heterogeneity index; *Calculated according to inverse-variance random-effects model. P-values in bold considered significant.

Leave-one-out sensitivity test for the meta-analysis assessing Total Cholesterol and all AVF failure

Study Excluded	No. Studies	N_{Cases}	$N_{Controls}$	SMD (95%CI)	p value*	I^2	I^2 p value
Nil	4	192	464	0.14 [-0.12, 0.41]	0.28	50%	0.11
Candan	3	150	426	0.14 [-0.22, 0.50]	0.45	67%	**0.05**
Gagliardi	3	155	410	0.07 [-0.24, 0.39]	0.67	58%	0.09
Kaygin	3	117	153	0.08 [-0.30, 0.47]	0.67	59%	0.08
Kirkpantur	3	154	403	0.27 [0.07, 0.46]	**0.007**	0%	0.73

SMD: standardised mean difference; I^2: heterogeneity index; *Calculated according to inverse-variance random-effects model. P-values in bold considered significant.

Leave-one-out sensitivity test for the meta-analysis assessing Triglycerides and all AVF failure

Study Excluded	No. Studies	N_{Cases}	$N_{Controls}$	SMD (95%CI)	p value*	I^2	I^2 p value
Nil	7	322	590	-0.02 [-0.17, 0.12]	0.74	0%	0.70
Bilgic	6	271	547	-0.01 [-0.17, 0.14]	0.86	0%	0.60
Bojakowski	6	311	556	-0.03 [-0.18, 0.12]	0.68	0%	0.61
Candan	6	280	552	-0.04 [-0.20, 0.11]	0.58	0%	0.67
Kaygin	6	247	279	-0.00 [-0.18, 0.17]	0.96	0%	0.60
Kirkpantur	6	284	529	0.01 [-0.14, 0.17]	0.88	0%	0.84
Wu	6	281	531	-0.06 [-0.21, 0.09]	0.44	0%	0.82
Yilmaz	6	258	546	-0.02 [-0.18, 0.13]	0.78	0%	0.58

SMD: standardised mean difference; I^2: heterogeneity index; *Calculated according to inverse-variance random-effects model. P-values in bold considered significant.

Leave-one-out sensitivity test for the meta-analysis assessing Albumin and AVF stenosis

Study Excluded	No. Studies	N_{Cases}	$N_{Controls}$	SMD (95%CI)	p value*	I^2	I^2 p value
Nil	7	257	311	-0.24 [-0.57, 0.08]	0.14	71%	**0.002**
Bilgic	6	206	268	-0.28 [-0.67, 0.11]	0.17	75%	**0.001**
Bojakowski	6	246	277	-0.14 [-0.42, 0.15]	0.34	60%	**0.03**
Jaberi	6	239	271	-0.32 [-0.66, 0.03]	0.07	72%	**0.003**
Kim	6	223	281	-0.28 [-0.66, 0.09]	0.14	75%	**0.001**
Kirkpantur	6	219	250	-0.13 [-0.42, 0.16]	0.39	56%	**0.04**
Wu	6	216	252	-0.28 [-0.67, 0.11]	0.16	75%	**0.001**
Yilmaz	6	193	267	-0.30 [-0.68, 0.07]	0.12	73%	**0.002**
Nil †	6	246	277	-0.14 [-0.42, 0.15]	0.34	60%	**0.03**
Bilgic †	5	195	234	-0.14 [-0.49, 0.21]	0.43	68%	**0.01**
Jaberi †	5	228	237	-0.20 [-0.50, 0.11]	0.20	62%	**0.03**
Kim †	5	212	247	-0.15 [-0.49, 0.18]	0.37	67%	**0.02**
Kirkpantur †	5	208	216	-0.01 [-0.20, 0.19]	0.93	0%	0.87
Wu †	5	205	218	-0.14 [-0.49, 0.21]	0.42	68%	**0.01**
Yilmaz †	5	182	233	-0.18 [-0.51, 0.16]	0.31	65%	**0.02**

SMD: standardised mean difference; I^2: heterogeneity index; *Calculated according to inverse-variance random-effects model. P-values in bold considered significant. †Analysis excluded mixed population data from Bojakowski *et al*.

Leave-one-out sensitivity test for the meta-analysis assessing Albumin and AVF thrombosis

Study Excluded	No. Studies	N_{Cases}	$N_{Controls}$	SMD (95%CI)	p value*	I^2	I^2 p value
Nil	3	90	126	-0.53 [-1.14, 0.08]	0.09	77%	**0.01**
Bojakowski	2	79	92	-0.26 [-0.78, 0.25]	0.31	64%	0.09
Candan	2	48	88	-0.81 [-1.50, -0.13]	**0.02**	64%	0.10
Gagliardi	2	53	72	-0.58 [-1.79, 0.63]	0.35	88%	**0.004**

SMD: standardised mean difference; I^2: heterogeneity index; *Calculated according to inverse-variance random-effects model. P-values in bold considered significant.

Leave-one-out sensitivity test for the meta-analysis assessing CRP and AVF stenosis

Study Excluded	No. Studies	N_{Cases}	$N_{Controls}$	SMD (95%CI)	p value*	I^2	I^2 p value
Nil	5	201	210	0.19 [-0.14, 0.52]	0.26	61%	**0.04**
Bilgic	4	150	167	0.17 [-0.25, 0.59]	0.42	68%	**0.02**
Bojakowski	4	190	176	0.06 [-0.15, 0.26]	0.59	0%	0.44
Kim	4	167	180	0.26 [-0.16, 0.67]	0.23	70%	**0.02**
Wu	4	160	151	0.29 [-0.09, 0.68]	0.13	61%	0.06
Yilmaz	4	137	166	0.25 [-0.20, 0.69]	0.27	70%	**0.02**
Nil †	4	190	176	0.06 [-0.15, 0.26]	0.59	0%	0.44
Bilgic †	3	139	133	-0.03 [-0.27, 0.21]	0.79	0%	0.73
Kim †	3	156	146	0.07 [-0.19, 0.33]	0.59	23%	0.27
Wu †	3	149	117	0.13 [-0.11, 0.38]	0.28	0%	0.54
Yilmaz †	3	126	132	0.05 [-0.24, 0.34]	0.72	26%	0.26

CRP: C-reactive protein; SMD: standardised mean difference; I^2: heterogeneity index; *Calculated according to inverse-variance random-effects model. P-values in bold considered significant. †Analysis excluded mixed population data from Bojakowski *et al*.

Leave-one-out sensitivity test for the meta-analysis assessing CRP and AVF thrombosis

Study Excluded	No. Studies	N_{Cases}	$N_{Controls}$	SMD (95%CI)	*p* value*	I^2	I^2 *p* value
Nil	4	150	207	0.27 [-0.08, 0.62]	0.13	57%	0.07
Bojakowski	3	139	173	0.12 [-0.10, 0.34]	0.30	0%	0.83
Candan	3	108	169	0.38 [-0.08, 0.85]	0.11	67%	**0.05**
Gagliardi	3	113	153	0.33 [-0.18, 0.84]	0.21	72%	**0.03**
Ozdemir	3	90	126	0.37 [-0.17, 0.91]	0.18	70%	**0.04**
Nil †	3	139	173	0.12 [-0.10, 0.34]	0.30	0%	0.83
Candan †	2	97	135	0.16 [-0.10, 0.42]	0.24	0%	0.79
Gagliardi †	2	102	119	0.09 [-0.18, 0.35]	0.52	0%	0.68
Ozdemir †	2	79	92	0.11 [-0.19, 0.41]	0.47	0%	0.54

CRP: C-reactive protein; SMD: standardised mean difference; I^2: heterogeneity index; *Calculated according to inverse-variance random-effects model. P-values in bold considered significant.

†Analysis excluded mixed population data from Bojakowski *et al*.

S1 Table. Blood collection and laboratory methods used to quantify biomarkers

Reference	Blood Collection	Biomarkers	Quantification Method	Blood medium
Baumann [8]	Pre-operative	Fibrinogen	Automated analyser	n/r
Bilgic [9]	At Failure	Albumin	n/r	n/r
		Calcium	Automated analyser	Serum
		Phosphorus	Automated analyser	Serum
		PTH	n/r	n/r
		HDL-C	Automated analyser	Serum
		TG	Automated analyser	Serum
		LDL-C	Automated analyser	Serum
		Haemoglobin	n/r	n/r
		Transferrin Saturation	n/r	n/r
		Ferritin	n/r	n/r
		CRP	Automated analyser	Serum
		sE-Selectin	ELISA	Serum
		sEPCR	ELISA	Plasma
Bojakowski [10]	Peri-operative	CRP	Automated analyser	n/r
		Procalcitonin	Automated analyser	n/r
		HDL-C	Automated analyser	n/r
		LDL-C	Automated analyser	n/r
		TG	Automated analyser	n/r
		Creatinine	Automated analyser	n/r
		Albumin	Automated analyser	Serum
		Albumin corrected Calcium	Automated analyser	Serum
		WBC	Automated analyser	n/r
		Neutrophils	Automated analyser	n/r
		Lymphocytes	Automated analyser	n/r
		Monocytes	Automated analyser	n/r
		Hematocrit	Automated analyser	n/r
		MCV	Automated analyser	n/r
		MCH	Automated analyser	n/r
		RDW	Automated analyser	n/r
		Platelets	Automated analyser	n/r
		Iron	Automated analyser	n/r
		Transferrin	Automated analyser	n/r
		Ferritin	Automated analyser	n/r
		RBC	Automated analyser	n/r
Candan [11]	Functional fasted mid-week HD session	Ca x P	n/r	n/r
		PTH	n/r	n/r
		Haemoglobin	n/r	n/r
		WBC	n/r	n/r
		Platelets	n/r	n/r
		Total cholesterol	n/r	n/r
		TG	n/r	n/r
		LDL-C	n/r	n/r
		HDL-C	n/r	n/r
		Albumin	n/r	n/r
		CRP	n/r	n/r
		Ferritin	n/r	n/r
		Transferrin Saturation	n/r	n/r
		VEGF-A	ELISA	Plasma
Gagliardi [12]	Mean of annual blood results	TC	Col-esterase + oxidase POD method	Plasma
		Fibrinogen	Clauss method	Plasma
		CRP	Automated analyser	n/r
		MIS	n/r	n/4
		Albumin	n/r	Serum
		Anti-CMV IgG	Chemiluminescent immunoassay	Serum
		Anti-H. *pylori* IgG	ELISA	Serum
		Anti-C. *pneumoniae* IgG	Micro-immunofluorescent assay	Serum
Jaberi [13]	Unclear	Haemoglobin	Automated analyser	n/r
		Neutrophils	Automated analyser	n/r
		Albumin	Dichromatic digital endpoint method	n/r
		Calcium	Indirect potentiametry	n/r
		Phosphorus	A time-rated method	n/r
		Platelets	Automated analyser	n/r
		PTH	n/r	n/r
		Ferritin	n/r	n/r
		Ca-PO$_4$	n/r	n/r
Kaygin [14]	Peri-operative	TC	n/r	Serum
		HDL-C	n/r	Serum
		LDL-C	n/r	Serum
		TG	n/r	Serum
		Albumin	Automated analyser	Serum
		CRP	Nephelometry	Serum
		Fibrinogen	Automated analyser	Serum
Kim [15]	Mean over 6 months	Fetuin-A	ELISA	n/r
		OPG	ELISA	Serum
		Heatshock protein 70	ELISA	Serum
		Uric Acid	n/r	n/r
		Calcium	n/r	n/r
		Phosphorus	n/r	n/r

		Ca x P	n/r	n/r
		PTH	Immunoradiometric assay	n/r
		TG	Automated analyser	Serum
		TC	Automated analyser	Serum
		LDL-C	Automated analyser	Serum
		HDL-C	Automated analyser	Serum
		CRP	Automated analyser	Serum
		Albumin	Automated analyser	Serum
Kirkpantur [16]	Peri-operative	Haemoglobin	Ultraviolet assay	n/r
		Albumin	Ultraviolet assay	Serum
		CRP	Nephelometry	Serum
		TC	Ultraviolet assay	Serum+Plasma
		TG	Ultraviolet assay	Serum
		LDL-C	Friedewald formula	Serum
		HDL-C	Precipitation	Serum+Plasma
		Glucose	Glucose-oxidase method	Plasma
Masaki [17]	At failure	Hematocrit	n/r	n/r
Ozdemir [18]	6 months prior AVF failure	Hamatocrit	Automated analyser	n/r
		Eosinophil	Automated analyser	Whole blood
		Ferritin	Automated analyser	Serum
		CRP	Latex agglutination	Serum
		PTH	Automated analyser	Serum
Wu [19]	Peri-operative	LDL-C	Automated analyser	Plasma
		HDL-C	Automated analyser	Plasma
		TG	Automated analyser	Plasma
		Calcium	Automated analyser	Plasma
		Phosphorus	Automated analyser	Plasma
		Albumin	Automated analyser	Plasma
		Creatinine	Automated analyser	Plasma
		CRP	Automated analyser	Plasma
		Homocysteine	ELISA	Plasma
		ADMA	ELISA	Plasma
Yilmaz [20]	6 months prior to failure	Albumin	n/r	Serum
		Calcium	n/r	Serum
		Phosphorus	n/r	Serum
		Ca x P	n/r	Serum
		PTH	n/r	n/r
		HDL-C	n/r	Serum
		LDL-C	n/r	Serum
		Haemoglobin	n/r	Serum
		Transferrin saturation	n/r	n/r
		Ferritin	n/r	n/r
		CRP	n/r	n/r
		Uric acid	n/r	n/r
		WBC	n/r	n/r
		NLR	n/r	n/r

PTH: parathyroid hormone; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; CRP: C-reactive protein; sE-Selectin: soluble E-selectin; eEPCR: soluble endothelial protein C receptor; TG: triglycerides; WBC: white blood cell count; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; RDW: red blood cell distribution width; RBC: red blood cell count; Ca x P: calcium times phosphate; VEGF-A: vascular endothelial growth factor A; MIS: malnutrition inflammation score; CMV: cytomegalovirus; OPG: osteoprotegerin; TC: total cholesterol; ADMA: asymmetrical dimethylarginine; NLR: neutrophil-lymphocyte ratio.

Appendix 9:

Rodríguez AJ*, Nunes V dos S*, Mastronardi CA, et al (2015) "Association between circulating adipocytokines concentrations and microvascular complications in patients with type 2 diabetes mellitus: a systematic review and meta-analysis of controlled cross-sectional studies". J Diabetes Complications. doi: 10.1016/j.jdiacomp.2015.11.004

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Association between circulating adipocytokine concentrations and microvascular complications in patients with type 2 diabetes mellitus: A systematic review and meta-analysis of controlled cross-sectional studies

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ARTICLE INFO

ABSTRACT

Background: The adipocytokines leptin and adiponectin have been variously associated with diabetic microvascular complications. No comprehensive clinical data exist examining the association between adipocytokines and the presence of these complications.
Methods: This is a systematic review of cross-sectional studies comparing circulating adipocytokines in patients with type 2 diabetes mellitus (T2DM), with and without microvascular complications. Studies were retrieved from MEDLINE, EMBASE, Scopus and Cochrane databases. Study quality was evaluated using a modified Newcastle–Ottawa Scale. Meta-analysis was performed using an inverse-variance model, providing standardised mean differences (SMD) and 95% confidence intervals (CI). Heterogeneity was determined by I^2 statistic.
Results: Amongst 554 identified studies, 28 were included in the review. Study quality range was 3.5–9 (maximum 11). Higher leptin levels were associated with microalbuminuria (SMD = 0.41; 95% CI = 0.14–0.67; n = 901; p = 0.0003), macroalbuminuria (SMD = 0.68; 95% CI = 0.30–1.06; n = 406; p = 0.0004), and neuropathy (SMD = 0.26; 95% CI = 0.07–0.44; n = 609; p = 0.008). Higher adiponectin levels were associated with microalbuminuria (SMD = 0.55; 95% CI = 0.29–0.81, n = 274; p < 0.001), macroalbuminuria (SMD = 1.37; 95% CI = 0.78–1.97, n = 246; p < 0.00001), neuropathy (SMD = 0.25; 95% CI = 0.14–0.36; n = 1516; p < 0.00001), and retinopathy (SMD = 0.38; 95% CI = 0.25–0.51; n = 1306; p < 0.00001). Meta-regression suggested no influence of body mass index and duration of diabetes on effect size, and a weak trend in terms of age on effect size.
Discussion: Our meta-analysis suggests leptin and adiponectin levels are higher in T2DM patients with microvascular complications. Studies were limited by cross-sectional design. Large prospective analyses are required to validate these findings.

1. Introduction

Diabetes mellitus (DM), a chronic progressive disease characterised by altered glucose homeostasis, is a significant cause of global morbidity

and mortality. In Australia, recent statistics suggest that approximately 3.61 million people are affected by diabetes mellitus, both type 1 and type 2 (Whiting, Guariguata, Weil, & Shaw, 2011). Based on studies from 110 countries, the International Diabetes Federation (IDF) estimated that in 2014 there were 387 million people with diabetes; this number is expected to rise to nearly 600 million by 2035 (IDF, 2014).

Due to chronic hyperglycaemia and associated metabolic abnormalities, patients with DM are at an increased risk for developing several macro- and microvascular complications (Hammes, 2003), namely retinopathy, nephropathy and neuropathy. Retinopathy affects approximately 25% of the population with diabetes, and is associated with blood vessel damage in the retina, with the potential to cause significant, and sometimes irreversible, vision loss (Klein,

Klein, Moss, Davis, & DeMets, 1984). Nephropathy has a prevalence of approximately 30% in patients with diabetes; it is associated with vascular injury in the nephrons, affecting renal function at different levels. Serious nephropathy can evolve into chronic kidney disease and eventual end-stage renal disease, meaning these patients must either enter long-term haemodialysis or require renal transplantation (Costacou, Ellis, Fried, & Orchard, 2007). Neuropathy is associated with vascular and neuronal damage (commonly peripheral, but also autonomic), leading to impairment of sensation, movement or organ/gland control. In more severe cases, impairment of sensation can result in ulceration requiring limb amputation. Within the population with diabetes, neuropathy has a prevalence as high as 25% (Dyck et al., 1993; Edwards, Vincent, Cheng, & Feldman, 2008).

Whilst the clinical aspects of these microvascular complications are well understood, their molecular mechanisms remain unclear. As type 2 diabetes mellitus (T2DM) is commonly associated with obesity, it has been proposed that adipocytokines, which are produced by the adipose tissue, can directly influence the pro-atherogenic and pro-inflammatory environment of the vascular walls (Cha et al., 2012). Leptin and adiponectin are the two most abundant adipocytokines, and have previously been linked with vascular complications in patients with T2DM (Hanai et al., 2010, 2014; Matsuda, Kawasaki, Inoue, et al., 2004; Matsuda, Kawasaki, Yamada, et al., 2004; Stehouwer, Lambert, Donker, & van Hinsbergh, 1997; Uckaya et al., 2000).

Leptin is a 16-kDa molecule synthesised primarily by the adipose tissue. It is the chief energy regulatory hormone, acting both centrally in the hypothalamus at the arcuate nucleus to produce sensations of satiety; and peripherally to stimulate fatty acid oxidation and glucose disposal (Paz-Filho et al., 2012). Furthermore, leptin has several cytokine-like actions. Importantly, leptin shares structural homology with the interleukin-6 (IL-6) molecule, which is a well-established pro-inflammatory effector (Mak, Cheung, Cone, & Marks, 2006). Leptin too has emerged as an important inflammatory molecule responsible for vascular inflammation, increased oxidative stress, endothelial dysfunction and proliferation of vascular smooth muscle cells (VSMC), and resultant intimal hyperplasia (Guzik, Marvar, Czesnikiewicz-Guzik, & Korbut, 2007).

Adiponectin is also an adipocytokine, but its function is complementary and antagonistic to leptin (Safai et al., 2015). For instance, whilst leptin promotes VSMC proliferation, *in vitro* studies indicate that adiponectin has the inverse effect and furthermore, adiponectin has been shown to inhibit TNF-α, an important inflammatory mediator which facilitates cell adhesion (Chandran, Phillips, Ciaraldi, & Henry, 2003; Okamoto et al., 2002; Yamauchi et al., 2001). Therefore, these adipocytokines are of direct clinical relevance to diabetic microvascular complications.

In patients with diabetes and obesity, leptin levels are increased and adiponectin, decreased (Goldstein, Scalia, & Ma, 2009; Havel, 2004). In this way, patients with T2DM may be exposed to an increased risk of leptin/adiponectin axis-mediated vascular inflammation, and subsequent damage leading to the development of microvascular complications (Niswender & Magnuson, 2007; Payne, Tune, & Knudson, 2014).

Despite several studies demonstrating the pathophysiological association between these adipocytokines and endothelial dysfunction (Van de Voorde, Pauwels, Boydens, & Decaluwé, 2013), clinical data have proved heterogeneous, and no study to date has systematically examined the evidence in this area. Consequently, we sought to survey the literature with the aim to (1) through a systematic review, synthesise and critically evaluate work done in this field and (2) through meta-analysis, clarify the results from this literature, to determine if there is an association between the adipocytokines leptin and adiponectin, and diabetic microvascular complications.

2. Methods

2.1. Study focus and eligibility criteria

To achieve our aims, we conducted a systematic review in accordance with the PRISMA guidelines (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) (Moher, Liberati, Tetzlaff, & Altman, 2009) and sought original (observational) studies that examined the association between circulating concentrations of leptin, adiponectin or both (measured by any kind of assay), with the presence or absence of diabetic microvascular complications (retinopathy, nephropathy and neuropathy). Studies were considered eligible for systematic review if the investigation evaluated either concentrations of leptin or adiponectin (or both) in patients with T2DM whom were affected by at least one microvascular complication, and compared these concentrations against patients with T2DM without these complications.

Specific exclusion criteria were: studies not comparing two groups of patients with T2DM (e.g. diabetic complications versus non-diabetic complications), studies evaluating only a cohort of patients with type 1 diabetes, studies that did not present data that could be interpreted as a comparison of leptin or adiponectin between patients with and without microvascular complications, animal or cell-based studies, and individual case reports.

The diagnosis of T2DM was made according to the American Diabetes Association (ADA) guidelines (ADA, 2010). Further, in accordance with these guidelines, patients with urinary albumin excretion between 30 and 300 mg/day (or if urinary albumin excretion between 30 and 300 μg/g creatinine) were considered to have microalbuminuria; macroalbuminuria was defined as urinary albumin excretion >300 mg/day (or if urinary albumin excretion >300 mg/g creatinine), and normoalbuminuria was defined as urinary albumin excretion <30 mg/day (or if urinary albumin excretion <30 μg/g creatinine; i.e., absence of nephropathy) (Group, 2013). Diabetic retinopathy (DR) was classified as normal, non-proliferative and proliferative according to fundoscopic exam performed by an ophthalmologist. Non-proliferative DR was defined based on one or more of the following findings: exudates, microvascular abnormalities, microaneurysm, or haemorrhage. Proliferative DR was diagnosed in the presence of new vessels. For the diagnosis of peripheral or autonomic neuropathies, we considered only validated methods to be appropriate. Peripheral neuropathy was defined as patient reporting changes in sensations (subjectively, or objectively by employing validated screening instruments such as the Michigan Neuropathy Screening Instrument) (Jung, Kim, Mok, Kang, & Kim, 2014), altered clinical tests (monofilament test, vibration test and ankle reflex test) and/or abnormal electrophysiological studies. Autonomic neuropathy was diagnosed by abnormal cardiovascular reflex tests (Jung et al., 2012).

2.2. Units of measurement

Leptin levels were recorded as ng/mL. In order to have unit consistency across all the studies examining adiponectin, we applied a conversion factor of 0.03 to adiponectin results that were not reported as μg/mL. This was determined by contacting the manufacturer of the assay used in one study (Matsuda, Kawasaki, Inoue, et al., 2004; Matsuda, Kawasaki, Yamada, et al., 2004) that measured the 30 kDa form of adiponectin (personal correspondence).

2.3. Information sources and search strategy

We surveyed the MEDLINE (archives from 1966 to 2014), Scopus (1996–2014), EMBASE (1947–2014) and Cochrane Library (1992–2014) databases, and applied the following title/abstract terms ["leptin" OR "adiponectin"] AND ["retinopathy" OR "nephropathy" OR "neuropathy" OR "microvascular complication"] with no language restriction, on the 16th of August 2014. Additionally, we manually scanned the reference lists of eligible texts and the related articles lists that were generated, following a database search for other potential studies of interest. We termed these texts the "grey literature".

For eligible studies unavailable online, attempts were made to obtain full text manuscripts direct from authors and further data were also sought direct from authors in order to ensure study eligibility.

2.4. Meta-analysis eligibility

Studies were eligible for meta-analysis if they first satisfied the criteria for systematic review and reported data specifically relating mean leptin and/or adiponectin (serum or plasma) concentrations in a group of patients with at least one microvascular complication (case), against data relating mean leptin and/or adiponectin (serum or plasma) concentrations in a group of patients without microvascular complications (control). Studies were excluded from meta-analysis if the study did not specifically report leptin and/or adiponectin concentrations in patients with and without a specific microvascular complication (for example, a cohort comprising a mix of patients with retinopathy, patients with nephropathy and patients with neuropathy). Contact was made with authors requesting these specific data in an attempt to include as much literature as possible.

2.5. Study selection and data extraction process

Two reviewers (AJR and VSN) independently screened the records identified by the literature search for studies eligible for full-text review. Both reviewers extracted data using an extraction template. Information was compiled relating to: study design; study participants and baseline risk factors; outcome measurement and assessment; patient biochemical data (specifically of the adipocytokines of interest); statistical analysis of results; limitations highlighted by study investigators; and limitations not considered by investigators. For eligible cohort studies, data were extracted from baseline evaluation. Any discrepancies were resolved through iteration and consensus on the final output.

2.6. Quality assessment

In addition to data extraction, two reviewers (GP-F and CAM) independently performed a quality assessment of the studies included following screening. As no standardised quality assessment tool exists for investigations assessing adipocytokines in diabetic microvascular complications, we modified the previously validated Newcastle–Ottawa Quality Assessment Scale (scale widely used for quality assessment of observational studies), to suit our aims (Wells et al., 2009). In a "star rating system", each included study was judged in the following areas: sample representativeness; sample size; outcome definition and measurement; comparability of results; outcome assessment and statistical methods. For the final scoring, the average score given by both reviewers was calculated. A sample data extraction form and quality assessment tool is provided (Supplementary Material A.1).

2.7. Meta-analysis

Data were first tabulated into a format that allowed comparison of mean leptin and adiponectin concentrations in groups of patients with and without microvascular complications. As leptin and adiponectin levels can be influenced by renal insufficiency, we performed the adipocytokine analysis considering levels of albuminuria: microalbuminuria versus normoalbuminuria; macroalbuminuria versus normoalbuminuria; macroalbuminuria versus microalbuminuria. For diabetic retinopathy, the group analyses were: diabetic retinopathy (non-proliferative and proliferative) versus no retinopathy; non-proliferative retinopathy versus no diabetic retinopathy; proliferative versus non-proliferative. Neuropathy was evaluated as neuropathy or no neuropathy. Tabulated outcomes were then synthesised using the inverse-variance method, and as adipocytokines were measured in either plasma or serum, standardised mean differences (SMD) was used instead of mean difference. The 95% confidence interval (95% CI) was also reported. Heterogeneity was determined by the Q statistic (defined as the I^2 statistic and its degrees of freedom) and inconsistency percentage (I^2) was defined as the ratio of the difference between the Q statistic and degrees of freedom with the Q statistic "$[(Q - df)/Q] \times 100$" (Higgins & Thompson, 2002). Random or fixed effects models were applied based on whether I^2 was greater than 50% in each analysis. To assess any impact important confounders might have on the effect size, we also conducted meta-regression where effect sizes were regressed against study-level confounders, and trends were noted giving an indication whether confounder was related to the effect size. All statistical operations were performed in consultation with a statistician (TN) and using the software RevMan v5.3 (The Nordic Cochrane Centre, The Cochrane Collaboration 2012).

2.8. Sensitivity analysis

In instances of a significant result in meta-analysis, to assess if one particular study contributed exceptionally to the result, we performed a leave-one-out sensitivity analysis. The meta-analysis was repeated with the exclusion of a single study, and was repeated for all the studies in the analysis (each time re-including the one study left out).

3. Results

3.1. Included literature

Initial database search identified 721 records, of which 167 were duplicates (i.e. appearing in more than one searched database), leaving 554 unique records available for abstract screening. Screening of abstracts removed a further 509 studies. This left 45 articles for full text review and of these, a further 17 studies were excluded for different reasons. Overall, 28 studies (Asakawa, Tokunaga, & Kawakami, 2001; Cha et al., 2012; Chan et al., 2004; Choe et al., 2013; Chung, Tsai, Chang, Shin, & Lee, 2005; Dossarps et al., 2014; Fruehwald-Schultes et al., 1999; Fujita et al., 2006; Gottsater, Ahren, & Sundkvist, 1999; Hanai et al., 2010; Jung et al., 2012, 2014; Kato et al., 2008; Komaba et al., 2006; Kopeisy, A. H. A., & Wasfy, 2011; Koshimura et al., 2004; Matsuda, Kawasaki, Inoue, et al., 2004; Matsuda, Kawasaki, Yamada, et al., 2004; Parveen & Zia Qureshi, 2013; Pradeepa et al., 2015; Ran et al., 2010; Saito et al., 2007; Sari, Balci, & Apaydin, 2010; Swellam, Sayed Mahmoud And, & Ali, 2009; Uckaya et al., 2000; Wilson, Nelson, Nicolson, & Pratley, 1998; Yilmaz et al., 2004, 2008) satisfied our inclusion and exclusion criteria for systematic review, and data from 21 of those studies were eligible for meta-analysis (Asakawa et al., 2001; Cha et al., 2012; Chan et al., 2004; Choe et al., 2013; Chung et al., 2005; Dossarps et al., 2014; Fruehwald-Schultes et al., 1999; Fujita et al., 2006; Hanai et al., 2010; Jung et al., 2012, 2014; Kato et al., 2008; Komaba et al., 2006; Kopeisy et al., 2011; Koshimura et al., 2004; Matsuda, Kawasaki, Inoue, et al., 2004; Matsuda, Kawasaki, Yamada, et al., 2004; Parveen & Zia Qureshi, 2013; Pradeepa et al., 2015; Ran et al., 2010; Saito et al., 2007; Sari et al., 2010; Swellam et al., 2009; Uckaya et al., 2000; Wilson et al., 1998; Yilmaz et al., 2004, 2008) (Fig. 1). Included studies are listed in Table 1. Funnel plots are also provided (Supplementary Fig. A.1–A.9), which are mostly symmetrical, as an indication into the lack of publication bias.

3.2. Study characteristics

All 28 studies were cross-sectional analyses, twelve studies assessed retinopathy (Asakawa et al., 2001; Choe et al., 2013; Dossarps et al., 2014; Jung et al., 2014; Kato et al., 2008; Matsuda, Kawasaki, Inoue, et al., 2004; Matsuda, Kawasaki, Yamada, et al., 2004; Parveen & Zia Qureshi, 2013; Pradeepa et al., 2015; Sari et al., 2010; Swellam et al., 2009; Uckaya et al., 2000; Yilmaz et al., 2004); twenty studies assessed nephropathy (Asakawa et al., 2001; Cha et al., 2012; Chan et al., 2004; Choe et al., 2013; Chung et al., 2005; Fruehwald-Schultes et al., 1999; Fujita et al., 2006; Hanai et al., 2010; Jung et al., 2014; Kato
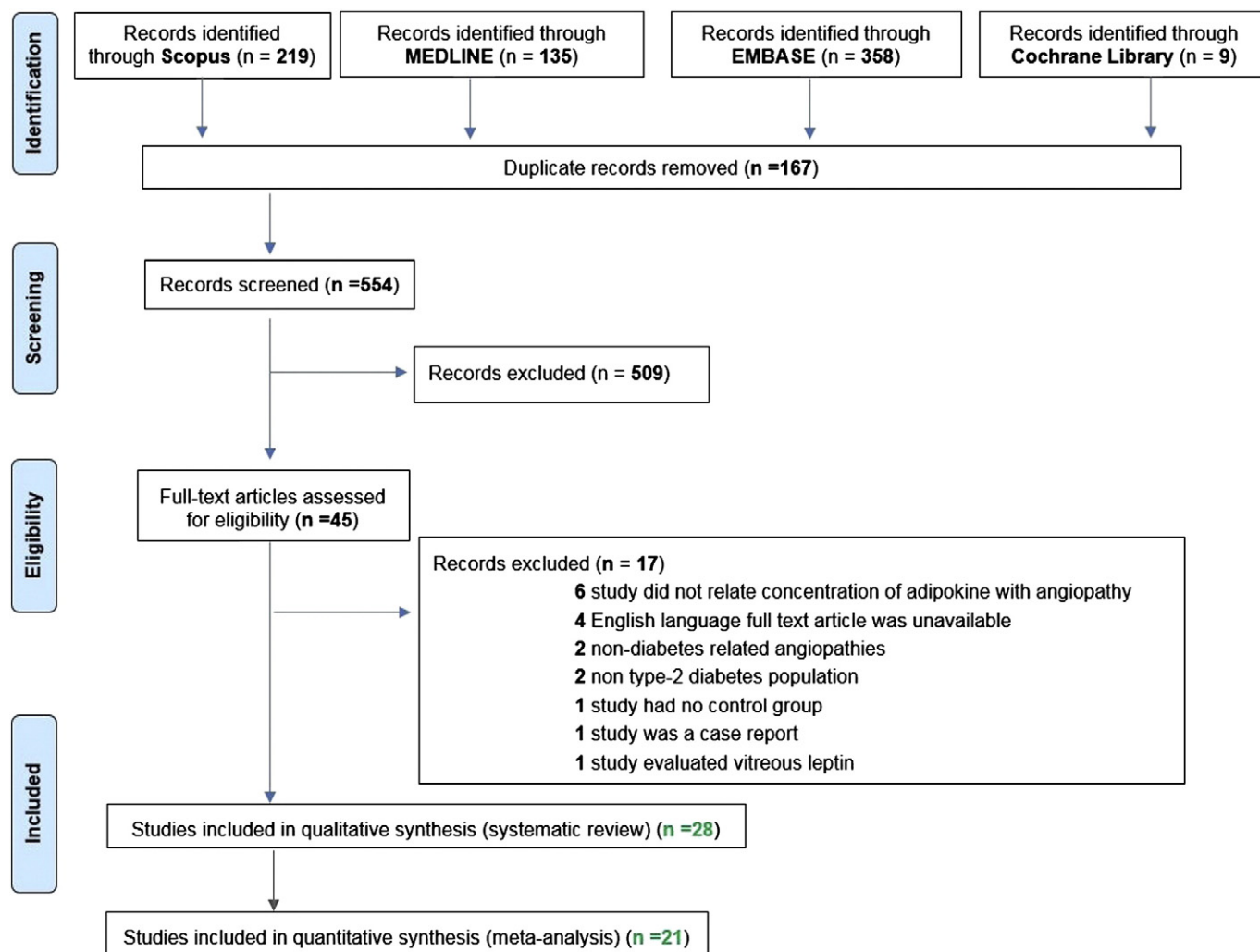
Fig. 1. PRISMA flow diagram of literature search protocol.

et al., 2008; Komaba et al., 2006; Kopeisy et al., 2011; Koshimura et al., 2004; Matsuda, Kawasaki, Inoue, et al., 2004; Matsuda, Kawasaki, Yamada, et al., 2004; Ran et al., 2010; Saito et al., 2007; Sari et al., 2010; Swellam et al., 2009; Wilson et al., 1998; Yilmaz et al., 2008) and eight studies examined neuropathy (Asakawa et al., 2001; Choe et al., 2013; Gottsater et al., 1999; Jung et al., 2012, 2014; Matsuda, Kawasaki, Inoue, et al., 2004; Matsuda, Kawasaki, Yamada, et al., 2004; Sari et al., 2010; Swellam et al., 2009). Nine studies assessed leptin from serum (Asakawa et al., 2001; Chan et al., 2004; Dossarps et al., 2014; Fruehwald-Schultes et al., 1999; Hanai et al., 2010; Jung et al., 2012, 2014; Kopeisy et al., 2011; Parveen & Zia Qureshi, 2013; Sari et al., 2010); seven studies measured plasma leptin (Cha et al., 2012; Chung et al., 2005; Gottsater et al., 1999; Matsuda, Kawasaki, Inoue, et al., 2004; Matsuda, Kawasaki, Yamada, et al., 2004; Sari et al., 2010; Uckaya et al., 2000; Wilson et al., 1998). Of the studies that assessed adiponectin, eight studies assessed serum adiponectin (Dossarps et al., 2014; Fujita et al., 2006; Jung et al., 2012, 2014; Kato et al., 2008; Koshimura et al., 2004; Pradeepa et al., 2015; Saito et al., 2007) and seven assessed plasma adiponectin (Choe et al., 2013; Matsuda, Kawasaki, Inoue, et al., 2004; Matsuda, Kawasaki, Yamada, et al., 2004; Ran et al., 2010; Swellam et al., 2009; Yilmaz et al., 2004, 2008) (Tables 1 and 2).

3.3. Quality assessment

Average quality assessment scores ranged from 3.5 to 9. Items #1, 3, 5 and 6 were the most reported items. These items related to sample representativeness (#1); outcome definition and measure-

ment (#3); outcome assessment (#5) and employment of appropriate statistical methods (#6). Items #2 and #4 respectively relating to sample size and comparability of results were variously reported. Table 3 summarises the individual scores.

3.4. Patient profile

Study population cohorts ranged from n = 30 participants to n = 1480 (Chung et al., 2005; Fruehwald-Schultes et al., 1999). Mean age of study participants ranged from 45.9 to 70.4 years-old in case groups, and 45.8 to 67.2 years-old in control groups (Cha et al., 2012; Ran et al., 2010). Mean patient body mass index (BMI) ranged from 23.7 to 35.8 kg/m^2 in cases (Komaba et al., 2006; Parveen & Zia Qureshi, 2013) and 23.2 to 33.7 kg/m^2 in controls (Dossarps et al., 2014; Koshimura et al., 2004). Finally, mean diabetes duration ranged from 1.31 to 18.7 years in cases (Fruehwald-Schultes et al., 1999; Uckaya et al., 2000), and 1.2 to 13.9 years in controls (Fruehwald-Schultes et al., 1999; Uckaya et al., 2000). These data and further details on patient risk factors are found in the Supplementary Tables (Tables A.1, A.2, A.3 and A.4).

3.5. Meta-analysis

3.5.1. Leptin

3.5.1.1. Retinopathy. Leptin concentration was not significantly different in patients with diabetic retinopathy (n = 92) relative to patients without diabetic retinopathy (n = 288) from three studies

Table 1
Details of studies included in the systematic review.

Study	Year	Patients (n)	Outcome	Blood medium		Meta-analysis
				Leptin	Adiponectin	
Asakawa	2000	182	Retinopathy, nephropathy, neuropathy	Serum	n/a	*
Cha	2012	163	Nephropathy	Plasma	n/a	*
Chan	2003	46	Nephropathy	Serum	n/a	*
Choe[a]	2013	693	Retinopathy, nephropathy, neuropathy	n/a	Plasma	*
Chung	2005	1480	Nephropathy	Plasma	n/a	*
Dossarps	2013	179	Retinopathy	Serum	Serum	*
Fruehwald-Schultes	1999	30	Nephropathy	Serum	n/a	*
Fujita	2006	53	Nephropathy	n/a	Serum	
Gottsater	1999	82	Neuropathy	Serum	n/a	
Hanai	2010	502	Nephropathy	Serum	n/a	*
Jung	2012	142	Neuropathy	Serum	Serum	*
Jung	2014	153	Retinopathy, nephropathy, neuropathy	Serum	Serum	*
Kato	2008	198	Retinopathy, nephropathy	n/a	Serum	
Komaba	2006	179	Nephropathy	n/a	n/r	*
Kopiesy	2011	100	Nephropathy	Serum	n/a	*
Koshimura	2004	38	Nephropathy	n/a	Serum	*
Matsuda	2004	231	Retinopathy, nephropathy	Plasma	Plasma	*
Matsuda	2004	105	Neuropathy	n/a	Plasma	
Parveen	2013	86	Retinopathy	Serum	n/a	*
Pradeepa	2014	487	Retinopathy	n/a	Serum	*
Ran	2010	50	Nephropathy	n/a	Plasma	*
Saito	2007	280	Nephropathy	n/a	Serum	
Sari	2010	157	Retinopathy, nephropathy, neuropathy	Plasma	n/a	*
Swellam	2009	179	Retinopathy, nephropathy, neuropathy	n/a	Plasma	
Uckaya	2000	136	Retinopathy	Plasma	n/a	*
Wilson	1999	20	Nephropathy	Plasma	n/a	
Yilmaz	2004	74	Retinopathy	n/a	Plasma	*
Yilmaz	2008	123	Nephropathy	n/a	Plasma	*

n/a = not applicable; n/r = not reported.

[a] Only provided biochemical data for nephropathy as study examined association between adiponectin gene variants and incidence of diabetic microvascular complications.

(Jung et al., 2014; Sari et al., 2010; Uckaya et al., 2000). Standardised mean difference (SMD) of the effect size was 0.49 and the 95% confidence interval (CI) ranged from −0.38 to 1.36 (p = 0.27) (Supplementary Fig. B.1). Heterogeneity for this analysis was 91% as determined by the I^2 index (Table 4). When considering severity of retinopathy, leptin was not significantly different in patients with non-proliferative diabetic retinopathy (n = 45) compared to patients without diabetic retinopathy (n = 153) [SMD = 0.78; 95% CI = −0.70 to 2.25; p = 0.30; I^2 = 93%] from two studies (Sari et al., 2010; Uckaya et al., 2000) (Supplementary Fig. B.2, Table 4). Leptin was not significantly different in patients with proliferative diabetic retinopathy (n = 29) compared to patients with non-proliferative diabetic retinopathy (n = 45) [SMD = 0.41; 95% CI = −0.11 to 0.93; p = 0.12; I^2 = 16%] from two studies (Sari et al., 2010; Uckaya et al., 2000) (Supplementary Fig. B.3, Table 4). Leptin was not significantly different in patients with proliferative diabetic retinopathy (n = 29) compared to patients without diabetic retinopathy (n = 153) [SMD = 0.91; 95% CI = −0.53 to 2.36; p = 0.21; I^2 = 90%] from two studies (Sari et al., 2010; Uckaya et al., 2000) (Supplementary Fig. B.4, Table 4).

3.5.1.2. Nephropathy. Leptin was significantly elevated in patients with microalbuminuria (n = 318) relative to patients with normoalbuminuria (n = 583) [SMD = 0.41; 95% CI = 0.14–0.67; p = 0.003; I^2 = 58%] from six studies (Cha et al., 2012; Chung et al., 2005; Fruehwald-Schultes et al., 1999; Hanai et al., 2010; Kopeisy et al., 2011; Sari et al., 2010) (Supplementary Fig. C.1, Table 4). A leave-one-out sensitivity analysis confirmed this result (Supplementary Fig. C.2). Leptin was significantly elevated in patients with macroalbuminuria (n = 157) relative to patients with normoalbuminuria (n = 249) [SMD = 0.68; 95% CI = 0.30–1.06; p = 0.0004; I^2 = 57%] from six studies (Cha et al., 2012; Chung et al., 2005; Fruehwald-Schultes et al., 1999; Kopeisy et al., 2011; Sari et al., 2010; Wilson et al., 1998) (Supplementary Fig. D.1, Table 4). A leave-one-out sensitivity analysis confirmed this result (Supplementary Fig D.2). Leptin was significantly elevated in patients with macroalbuminuria (n = 137) relative to patients with microalbuminuria (n = 150)

[SMD = 0.44; 95% CI = 0.20–0.68; p = 0.0003; I^2 = 0%] from four studies (Cha et al., 2012; Chung et al., 2005; Kopeisy et al., 2011; Sari et al., 2010) (Supplementary Fig. E, Table 4).

3.5.1.3. Neuropathy. Higher leptin concentration was observed in patients with diabetic neuropathy (n = 184) compared to patients without neuropathy (n = 425) [SMD = 0.26; 95% CI = 0.07–0.44; p = 0.008; I^2 = 0%] from four studies (Asakawa et al., 2001; Jung et al., 2012, 2014; Sari et al., 2010) (Supplementary Fig. F, Table 4).

3.5.2. Adiponectin

3.5.2.1. Retinopathy. Adiponectin was significantly elevated in patients with diabetic retinopathy (n = 324) compared to patients without diabetic retinopathy (n = 983) [SMD = 0.38; 95% CI = 0.25–0.51; p < 0.00001; I^2 = 0%] from three studies (Choe et al., 2013; Jung et al., 2014; Pradeepa et al., 2015) (Supplementary Fig. G.1, Table 4). When considering severity of retinopathy, adiponectin was not significantly different in patients with non-proliferative diabetic retinopathy (n = 221) compared to patients without diabetic retinopathy (n = 547) [SMD = −0.51; 95% CI = −2.45 to 1.43; p = 0.61; I^2 = 96%] from four studies (Dossarps et al., 2014; Kato et al., 2008; Pradeepa et al., 2015; Yilmaz et al., 2004) (Supplementary Fig. G.2, Table 4). Adiponectin did not differ significantly between patients with non-proliferative diabetic retinopathy (n = 43) relative to patients with proliferative diabetic retinopathy (n = 26) [SMD = −0.16; 95% CI = −1.52 to 1.20; p = 0.82; I^2 = 83%] from two studies (Kato et al., 2008; Yilmaz et al., 2004) (Supplementary Fig. D.3, Table 4).

3.5.2.2. Nephropathy. Adiponectin was significantly elevated in patients with microalbuminuria (n = 92) relative to patients with normoalbuminuria (n = 182) [SMD = 0.55; 95% CI = 0.29–0.81; p < 0.0001; I^2 = 0%] from four studies (Kato et al., 2008; Komaba et al., 2006; Koshimura et al., 2004; Ran et al., 2010) (Supplementary

Table 2
Outcome definition details of studies included in systematic review.

Study	Outcome	Outcome definition	Criterion	Case (n)	Controls (n)
Asakawa	Retinopathy	Retinopathy	n/r	39	139
	Nephropathy	Microalbuminuria	AER >30 mg/day	39	139
	Neuropathy	Peripheral neuropathy	Signs/symptoms of peripheral neuropathy	47	135
Cha	Nephropathy	Macroalbuminuria	AER ≥300 mg/day	76	40
		Microalbuminuria	AER 30–299 mg/day		
Chan	Nephropathy	Microalbuminuria	ACR >25 mg/mmol	34	12
Choe	Nephropathy	Microalbuminuria	ACR >30 mg/g	245	448
			AER >30 mg/day		
Chung	Nephropathy	Microalbuminuria	ACR 30–300 mg/g	113	50
		Macroalbuminuria	ACR >300 mg/g		
Dossarps	Retinopathy	Retinopathy	Fundoscopic examination	69	110
Fruehwald-Schultes	Nephropathy	Macroalbuminuria	AER >300 mg/day	20	20
		Microalbuminuria	AER 30–300 mg/day		
Fujita	Nephropathy	Microalbuminuria	ACR 30–299 mg/g	18	19
		Macroalbuminuria	ACR ≥300 mg/g	16	
Gottsater	Neuropathy	Parasympathetic	R-R interval variation	24	58
Hanai	Nephropathy	Microalbuminuria	ACR >25–355 mg/g (women); 17–250 mg/g (men)	158	344
Jung 2012	Neuropathy	Cardiac autonomic neuropathy	n/r	46	96
Jung 2014	Retinopathy	Retinopathy	Fundoscopic examination	18	135
	Nephropathy	Microalbuminuria	AER 20–200 μg/min; ACR 30–300 mg/g	20	133
		Macroalbuminuria	AER >200 μg/min		
			ACR >300 mg/g		
	Neuropathy	n/sp	Self-assessment	87	66
Kato	Retinopathy	Simple	Mild vascular changes	37	119
		Pre-proliferative	Ophthalmologist diagnosis	23	
		Proliferative	Ophthalmologist diagnosis	11	
	Nephropathy	Microalbuminuria	ACR 30–300 mg/g	47	116
		Macroalbuminuria	ACR ≥300 mg/g	24	
		Overt nephropathy	Serum creatinine ≥177 μmol/L	5	
Komaba	Nephropathy	Microalbuminuria	ACR 30–300 mg/g	93	86
		Macroalbuminuria	ACR ≥300 mg/g		
Kopiesy	Nephropathy	Microalbuminuria	AER 30–300 mg/day	20	60
		Macroalbuminuria	AER >300 mg/day		
Koshimura	Nephropathy	Microalbuminuria	ACR 30–300 mg/g	20	18
		Macroalbuminuria	ACR >300 mg/g		
Matsuda	Retinopathy	Proliferative retinopathy	Fundoscopic examination	191	263
	Nephropathy	Macroalbuminuria	ACR 3.4–33.9 g/mol	83	148
Matsuda	Neuropathy	Nerve conduction velocity	Motor and sensory nerve function	n/r	n/r
Pradeepa	Retinopathy	Proliferative retinopathy	Retinal photography	81	487
	Nephropathy	Microalbuminuria	n/r	143	
	Neuropathy	n/sp	n/r	138	
Parveen	Retinopathy	Proliferative retinopathy	Neovascularisation or pre-retinal haemorrhages	21	39
		Non-proliferative retinopathy	Microaneurysms, dot or blot haemorrhages, hard or soft exudates or venous beadings	26	
Ran	Nephropathy	Microalbuminuria	ACR 30–300 mg/day	32	18
		Macroalbuminuria	ACR >300 mg/day		
Saito	Nephropathy	Albuminuria	stage I (no microalbuminuria) to V (under dialysis treatment)	204	76
Sari	Retinopathy	Proliferative retinopathy	New vessels, confirmed by angiography, or previous treatment by photocoagulation	37	120
	Nephropathy	Microalbuminuria	AER 30–300 mg/day	39	119
		Macroalbuminuria	AER >300 mg/day		
	Neuropathy	Sensorial neuropathy	Clinically and/or with electromyography	51	263
		Autonomic neuropathy	Orthostatic test		
Swellam	Retinopathy	n/r	n/sp	71	n/r
	Nephropathy	n/r	Elevated serum creatinine level; stage V, under dialysis treatment	76	n/r
	Neuropathy	n/r	Self-assessment	110	n/r
Uckaya	Retinopathy	Proliferative retinopathy	Vascular abnormalities	37	33
Wilson	Nephropathy	Albuminuria	ACR ≥300 mg/day	10	10
Yilmaz	Retinopathy	Proliferative retinopathy	Vascular abnormalities	44	30
Yilmaz	Nephropathy	Microalbuminuria	Urinary protein >500 mg/day	45	40

ACR = albumin-creatinine ratio; AER = albumin excretion rate; mg = milligrams; n/r = not reported; mol = moles; g = grams; > = greater than; n/sp = not specified; μg = micrograms; min = minute.

Fig. H, Table 4). Adiponectin was significantly elevated in patients with macroalbuminuria (n = 64) relative to patients with normoalbuminuria (n = 182) [SMD = 1.37; 95% CI = 0.78–1.97; p < 0.00001; I^2 = 67%] from four studies (Kato et al., 2008; Komaba et al., 2006; Koshimura et al., 2004; Ran et al., 2010) confirmed in a sensitivity analysis (Supplementary Figs. I.1 and I.2; Table 4). Adiponectin was significantly elevated in patients with macroalbuminuria (n = 64) relative to patients with microalbuminuria (n = 92) [SMD = 0.87; 95%CI = 0.23, 1.51; p = 0.007; I^2 = 67%]

from four studies (Kato et al., 2008; Komaba et al., 2006; Koshimura et al., 2004; Ran et al., 2010) confirmed in a sensitivity analysis (Supplementary Figs. J.1 and J.2; Table 4).

3.5.2.3. Neuropathy. Adiponectin was significantly higher in patients with diabetic neuropathy (n = 506) relative to patients without diabetic neuropathy (n = 1010) [SMD = 0.25; 95% CI = 0.14–0.36; p < 0.00001; I^2 = 0%] from five studies (Choe et al., 2013; Jung et al.,

Table 3
Study quality scores using a modified Newcastle–Ottawa Scale.

Study	Item						Score
	1	2	3	4	5	6	
Asakawa 2001	**/**	*/–	**/**	*/**	**/**	*/*	9
Cha 2012	**/**	*/–	**/**	*/*	*/*	*/*	7.5
Chan 2004	*/**	–/–	**/**	–/*	*/*	*/*	6
Choe 2013	**/**	*/–	**/**	–/*	*/*	*/*	7
Chung 2005	***/**	*/–	**/**	**/*	*/–	*/*	8
Dossarps 2014	*/**	*/*	**/**	–/*	*/–	*/*	6.5
Fruehwald-Schultes 1999	*/*	–/–	**/**	*/*	*/**	*/*	6.5
Fujita 2006	**/*	–/–	**/**	**/*	*/*	*/*	7
Gottsater 1999	**/*	*/–	*/**	–/–	–/*	*/*	5
Hanai 2010	*/**	*/–	**/**	**/**	**/**	*/*	9
Jung 2012	*/*	*/–	**/**	*/**	**/**	*/*	8
Jung 2014	*/*	*/–	**/**	**/**	**/**	*/*	8.5
Kato 2008	**/*	*/–	**/**	**/**	**/**	*/*	9
Komaba 2006	**/**	*/*	**/**	*/*	*/–	*/–	7
Kopeisy 2011	*/**	–/*	**/**	–/**	*/–	*/*	6.5
Koshimura 2004	*/*	–/–	*/**	–/–	*/*	*/*	4.5
Matsuda 2004	*/*	*/–	**/**	*/**	*/**	*/*	7.5
Matsuda 2004	**/*	*/–	*/**	–/*	–/**	*/*	6
Pradeepa 2014	**/**	*/*	**/*	–/*	*/–	*/*	6.5
Parveen 2013	*/*	–/*	*/*	–/–	*/*	*/*	4.5
Ran 2010	**/*	–/*	**/**	–/*	*/–	*/*	6
Saito 2007	**/**	*/*	**/**	*/*	*/–	*/*	7.5
Sari 2010	**/**	*/*	**/**	–/*	*/–	*/*	7.5
Swellam 2009	*/**	*/*	*/*	*/*	*/–	*/*	6
Uckaya 2000	**/***	–/*	**/**	–/*	*/**	*/*	8
Wilson 1999	–/*	–/–	*/**	–/–	*/*	–/*	3.5
Yilmaz 2004	**/**	–/*	**/**	–/**	*/**	*/*	8
Yilmaz 2008	**/***	–/*	**/*	*/**	*/**	*/*	8.5

Scores were attributed by two reviewers, which were averaged to provide final scores. Itemised scoring criteria are provided in Supplementary Material A.1.

2012, 2014; Kato et al., 2008; Pradeepa et al., 2015) (Supplementary Fig. K, Table 4). The above results are summarised in Table 5.

3.5.3. Meta-regression

No relationship was found between BMI and duration of diabetes and effect sizes, however a slight trend was evident with respect to age (Supplementary Figs. L and M).

4. Discussion

4.1. Summary and highlight

Insulin-resistant T2DM affects a substantial proportion of the population, and is associated with debilitating microvascular complications such as retinopathy, nephropathy and neuropathy (Hammes, 2003). This investigation suggests that adipocytokines are relevant to microvascular disease

and represents the first aggregated study to demonstrate the association between increased leptin levels and nephropathy/neuropathy, and between increased adiponectin levels and nephropathy/neuropathy/retinopathy.

4.2. Role of leptin and adiponectin in MV complications

Adipocytokines (also called adipokines) are adipose tissue-derived molecules with hormone-like actions. Adipocytokines are established metabolic regulators with functions in several other systems including inflammation (Tian, Chang, Loh, & Hsieh, 2014). Leptin and adiponectin are two of the most abundant adipocytokines. Adiponectin levels are inversely related to the degree of adiposity (Cnop et al., 2003), whilst leptin increases with adiposity (Ostlund, Yang, Klein, & Gingerich, 1996).

Diabetic microvascular complications are principally driven by vascular inflammation, and there is strong evidence to suggest that adipocytokines have important functions in vascular inflammation

Table 4
Summary of heterogeneity of the studies included in the systematic review.

Adipocytokine	Analysis	# studies	n	Heterogeneity χ^2 (p value)	Inconsistency I^2 (%)	Model
Leptin	Micro vs Normo	6	901	12.03 (0.03)	58	Random
	Micro vs Macro	4	287	1.62 (0.66)	0	Fixed
	Macro vs Normo	6	406	11.62 (0.04)	57	Random
	DR vs No DR	3	380	21.45 (<0.0001)	91	Random
	Non DR vs no PDR	2	198	14.86 (0.0001)	93	Random
	PDR vs Non PDR	2	74	1.19 (0.28)	16	Fixed
	PDR vs No DR	2	182	10.25 (0.001)	90	Random
	Neuro vs No Neuro	4	609	2.76 (0.43)	0	Fixed
Adiponectin	Micro vs Normo	4	274	2.78 (0.43)	0	Fixed
	Macro vs Normo	4	246	9.06 (0.03)	67	Random
	Macro vs Micro	4	156	9.19 (0.03)	67	Random
	DR vs No DR	3	1307	1.85 (0.4)	0	Fixed
	Non PDR vs No DR	4	768	23.29 (<0.00001)	96	Random
	Non PDR vs PDR	2	69	5.8 (0.02)	83	Random
	Neuro vs No Neuro	5	1516	2.54 (0.64)	0	Fixed

Micro: microalbuminuria; Macro: macroalbuminuria; Normo: normoalbuminuria; DR: diabetic retinopathy; PDR: proliferative diabetic retinopathy; Neuro: neuropathy.

Table 5
Summary of results from the meta-analyses.

Adipokine	Analysis	n (total)	SMD (95% CI)	p value
Leptin	**Micro vs Normo**	**901**	**0.41 [0.14, 0.67]**	**0.0003**
	Macro vs Normo	**406**	**0.68 [0.30, 1.06]**	**0.0004**
	Macro vs Micro	**287**	**0.44 [0.20, 0.68]**	**0.0003**
	Neuro vs No Neuro	**609**	**0.26 [0.07, 0.44]**	**0.008**
	DR vs No DR	380	0.49 [−0.38, 1.36]	0.27
	Non PDR vs No DR	198	0.78 [−0.70, 2.25]	0.30
	PDR vs Non PDR	74	0.41 [−0.11, 0.93]	0.12
	PDR vs No DR	182	0.91 [−0.53, 2.36]	0.21
Adiponectin	**Micro vs Normo**	**274**	**0.55 [0.29, 0.81]**	**<0.0001**
	Macro vs Normo	**246**	**1.37 [0.78, 1.97]**	**<0.00001**
	Macro vs Micro	**156**	**0.87 [0.23, 1.51]**	**0.007**
	DR vs No DR	**1,306**	**0.38 [0.25, 0.51]**	**<0.00001**
	Non PDR vs No DR	768	−0.51 [−2.45, 1.43]	0.61
	Non PDR vs PDR	69	−0.16 [−1.52, 1.20]	0.82
	Neuro vs No Neuro	**1,516**	**0.25 [0.14, 0.36]**	**<0.00001**

Micro: microalbuminuria; Macro: macroalbuminuria; Normo: normoalbuminuria; DR: diabetic retinopathy; PDR: proliferative diabetic retinopathy; Neuro: neuropathy. Bold indicates significant result.

and endothelial dysfunction; leptin has deleterious actions on the vasculature, and adiponectin, has extensive vasculo-protective actions (Jamroz-Wiśniewska et al., 2014; Martínez-Martínez et al., 2014; Nevelsteen et al., 2013).

4.3. Comparing and contrasting this investigation with the literature

Adiponectin is generally considered to be an anti-inflammatory adipocytokine, and this is supported by a recent meta-analysis of over 3000 T2DM patients, where adiponectin was negatively correlated with diabetic retinopathy (Fan et al., 2014). This result is in contradiction to the present study. However, the population under consideration in the present study is considerably more heterogeneous, compared to the population included in the meta-analysis by Fan and colleagues, which was composed predominantly of Han Chinese. In considering that adiponectin concentration is influenced by genetic background, we postulate that our genetically diverse study represents the more robust result and may explain the disparate findings (Morimoto et al., 2014). Furthermore, it may be reasonable to hypothesise that adiponectin in fact plays no role in the pathophysiology of diabetic microvascular complications, and is increased in an attempt to counteract leptin's deleterious effects or may have a novel as yet unknown effect on diabetic microvascular complications (Costacou & Orchard, 2008; Ebert & Fasshauer, 2011). Overall, cellular and clinical evidence presents leptin and adiponectin as relevant and important to diabetic microvascular complications as these complications are driven by a component of inflammation in pathways which are directly affected by these adipocytokines.

Adiponectin levels differ in the presence of macrovascular versus microvascular complications. Patients with macrovascular complication display low concentrations of plasma adiponectin levels (Boyle, 2007; Wang, Gao, Su, Xu, & Fu, 2015; Yazici et al., 2012), whereas it has been reported that microvascular complications are associated with increased circulating adiponectin levels (Frystyk, Tarnow, Krarup Hansen, Parving, & Flyvbjerg, 2005). Atherosclerosis is an essential aetiological component of macrovascular complications (Fowler, 2011). The process of atherosclerosis appears to involve the synergistic action of hyperglycaemia and hyperlipidaemia (Chait & Bornfeldt, 2009). In an *in vivo* study, it was shown that diabetic pigs develop atherosclerosis in presence of hyperlipidaemia, but not in its absence, suggestive of a synergistic interaction between hyperlipidaemia and glycaemia in the progression of macrovascular complication (Gerrity, Natarajan, Nadler, & Kimsey, 2001). Thus, it could be hypothesised that increased hyperlipidaemia, which is ultimately involved in macrovascular complications, might play a role in

decreasing circulating adiponectin. Interestingly, low plasma adiponectin favours progression of atherosclerosis and vascular plaque formation (Broedl et al., 2009; Yazici et al., 2012). Therefore, the increased levels of circulating adiponectin observed in microvascular complications (as evident in this meta-analysis) could result from a compensatory mechanism to counter inflammatory processes that occur as part of the pathophysiology of microvascular disease complication, in the absence of macrovascular complications. In fact, a mounting body of evidence supports that adiponectin exerts vascular protective actions including anti-atherogenic effects (Broedl et al., 2009; Iwaki et al., 2003; Yazici et al., 2012).

Despite this literature, there has been little direct examination of the role of leptin and adiponectin in microvascular complications. We identified several studies that suggest leptin and adiponectin are relevant to these conditions (Asakawa et al., 2001; Cha et al., 2012; Chan et al., 2004; Choe et al., 2013; Chung et al., 2005; Dossarps et al., 2014; Fruehwald-Schultes et al., 1999; Fujita et al., 2006; Hanai et al., 2010; Jung et al., 2012, 2014; Kato et al., 2008; Komaba et al., 2006; Kopeisy et al., 2011; Koshimura et al., 2004; Matsuda, Kawasaki, Inoue, et al., 2004; Matsuda, Kawasaki, Yamada, et al., 2004; Parveen & Zia Qureshi, 2013; Pradeepa et al., 2015, Ran et al., 2010, Saito et al., 2007; Sari et al., 2010; Swellam et al., 2009; Uckaya et al., 2000; Wilson et al., 1998; Yilmaz et al., 2004, 2008). As this investigation attests, these studies have proven to be heterogeneous in their findings of associations between leptin and adiponectin and diabetic microvascular complications. In light of this, we have attempted to clarify these results by way of a systematic review and meta-analysis and found leptin and adiponectin to be significantly associated with diabetic microvascular complications.

4.4. Limitations of included studies

We identified some limitations of the included studies. All included studies were cross-sectional observational clinical studies. These studies leave open the possibility of selection bias as no randomisation was performed (recorded as part of the NOS). Therefore associations drawn in cross-sectional studies do not imply causation. Importantly, we did not exclude longitudinal studies from our literature search. In order to properly identify causative agents or assess the role of adipocytokines in microvascular complication development, large prospective trials are needed.

Another potential source of heterogeneity is the use of medications by the patients in this cohort. Generally, medication use was poorly reported and no instance exists where results (data relating to leptin and adiponectin with respect to patients with and without complications) were adjusted for medication use. Multiple co-morbidities are common in people with diabetes and many of these co-morbidities can be treated medically (e.g. fibrates for hypertriglyceridaemia) and these therapies are in addition to the many medical treatments patients with diabetes already receive for the diabetes itself (e.g. insulin, metformin or the glitazones). It is not known what effect these adjunct therapies have on adipocytokine levels in the blood in patients with microvascular complications.

Furthermore, several studies failed to report a method or criteria for evaluating diabetes in patients recruited, even if the presence of diabetes was part of the study inclusion criteria (Chan et al., 2004; Fruehwald-Schultes et al., 1999; Jung et al., 2012, 2014; Uckaya et al., 2000). This is important, as different countries have different standards for diabetes definitions. In considering that the studies included here were conducted in many different countries (e.g. Japan, Korea, Germany, Turkey and Egypt), we may be grouping some patients with poorly controlled diabetes together with patients with well-controlled diabetes according the definitions set in this country (Australia). Further to this, there was no direct assessment of possible leptin resistance in these patients. This is extremely relevant to the diabetes population as leptin resistance is a common finding in

patients with diabetes and obesity (Coppari & Bjørbæk, 2012). These effects may be mitigated by adjusting results according to baseline leptin levels, but this too was not performed in the studies included for meta-analysis.

There was also limited reporting of inflammatory markers which may be important in distinguishing the inflammatory effects of leptin and adiponectin from classic pro-inflammatory cytokines. Moreover, the duration of diabetes varied greatly and may have contributed to heterogeneity, as the risk of developing these chronic complications increases with longer diabetes duration. Finally, some studies were generally small in size, and as they included only patients with diabetes, the results may not be generalisable to patients with similar microvascular morbidities independent of diabetes.

4.5. Limitations of this meta-analysis

Our meta-analysis has some limitations. This meta-analysis was performed using aggregate data, and as such we did not have access to all primary data to enable adjustment of adipokine levels to factors strongly related to the risk of diabetic complications. However, we performed meta-regression analyses using the study-level confounders of age, BMI and duration of diabetes, representing the most important microvascular complication risk factors. No relationship between BMI and duration of diabetes was evident, suggesting that these study-level variables did not influence the effect size (SMD between leptin or adiponectin levels in case and control groups). A slight trend was evident with age: increasing age increased the effect size, suggesting that in patients with advanced age, the effect size (SMD between leptin or adiponectin levels in case and control groups) would be greater. However, it has been shown that the age-dependent changes in leptin and adiponectin levels are not very significant (Balaskó, Soós, Székely, & Pétervári, 2014).

In considering that leptin and adiponectin have been inconsistently associated with MV complications (some reporting no association, some reporting higher leptin or adiponectin to be associated and some reporting lower leptin or adiponectin to be associated), our study adds value to the literature in this area as a succinct, synthesised report. We cannot confirm whether the association of leptin with nephropathy is independent of hypertension. Increased leptin has variously been reported with normotension, hypertension and indeed may be elevated independent of hypertensive status (Almeida-Pititto, Gimeno, Freire, Ribeiro-Filho, & Ferreira, 2006; Tsuda & Nishio, 2004). Importantly, leptin does not mediate hypertension in human obesity and obesity is common amongst patients with T2DM suggesting increased leptin in nephropathy is occurring independently of hypertension (Brown, Meehan, & Gorden, 2015). However, to mitigate any potential effects, we attempted to include blood pressure in the meta-regression. As specific systolic or diastolic blood pressures were poorly reported by these studies, we sought these data directly from authors however we received no timely response. Other important sources of heterogeneity were sample sizes of some of the analyses, and that we grouped together results where adipocytokines were measured either in plasma or in serum. Also, ethnic and gender differences may account for possible bias in our results as we included studies from many racially and genetically distinct populations. Finally, we cannot discount any age or sex specific affects as possible sources of heterogeneity as for example, leptin levels tend to be higher in females.

5. Conclusion

Overall, we sought literature that examined the association between circulating levels of the adipocytokines leptin and adiponectin and diabetic microvascular complications. Our analysis revealed that higher leptin and adiponectin levels were associated with macro- and microalbuminuria and neuropathy. Further, elevated

adiponectin was also associated with retinopathy. This meta-analysis represents the first comprehensive quantitative assessment of adipocytokines in diabetic microvascular complications, and the results here could be translated into diagnostics, surveillance tools or for risk prediction uses. This pool of participants is likely underpowered to determine the influence of adipocytokines in microvascular complication development, and therefore large prospective studies are required to confirm and validate these findings independent of known risk factors and determine their causality.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jdiacomp.2015.11.004.

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Supplementary Figure A.1. Funnel plot of the analysis Leptin: Macroalbuminuria versus Normoalbuminuria.

Note
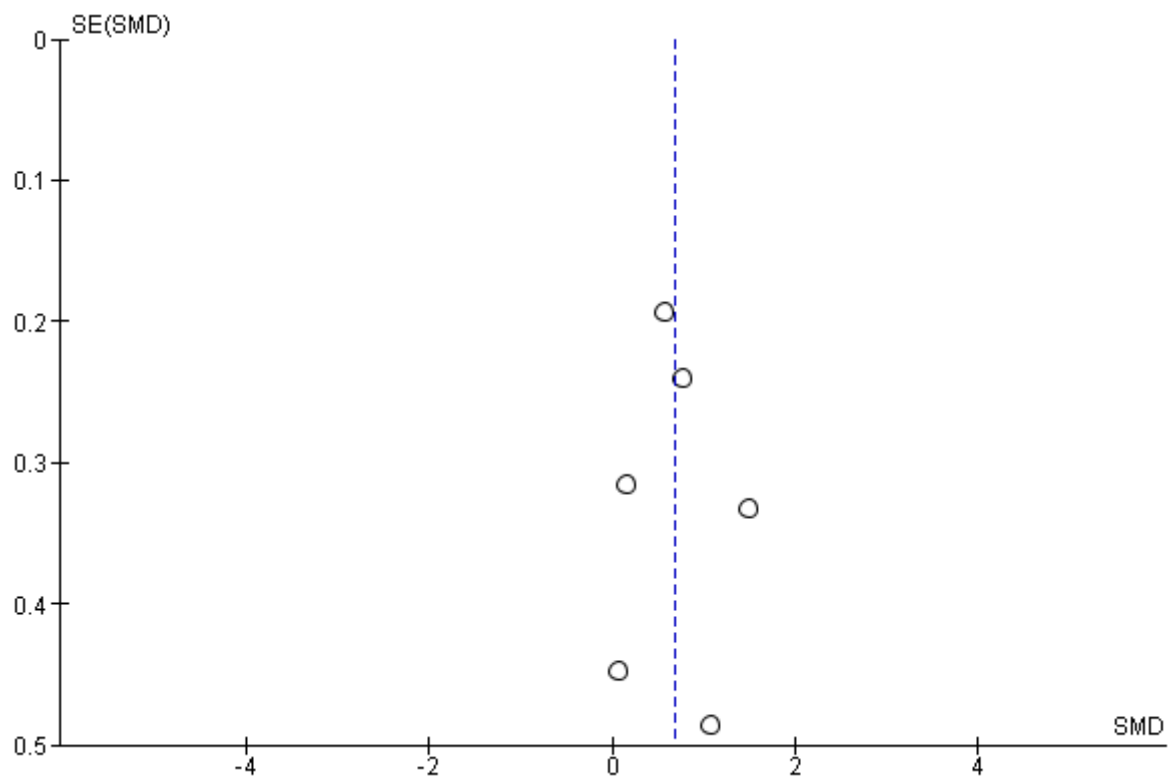
Note: The effect size (difference in mean leptin or adiponectin concentrations between case and control groups) is plotted on the horizontal axis against the standard error for the effect estimate. Standard error is used on the vertical axis instead of population size because the statistical power of a trial is determined by many factors and not solely sample size. For example, a study with 100,000 participants and 10 events is less likely to show a statistically significant intervention effect than a study with 1,000 participants and 100 events. The standard error provides a summary for these other factors. Plotting the standard error on the vertical axis places the larger, or most powerful, studies towards the top of the plot. Further, with this strategy, a simple triangular region can be plotted, within which 95% of studies would be expected to lie in the absence of both biases and heterogeneity. Overall, a symmetrical plot indicates minimal publication bias.

Supplementary Figure A.2. Funnel plot of the analysis Leptin: Macroalbuminuria versus Microalbuminuria.
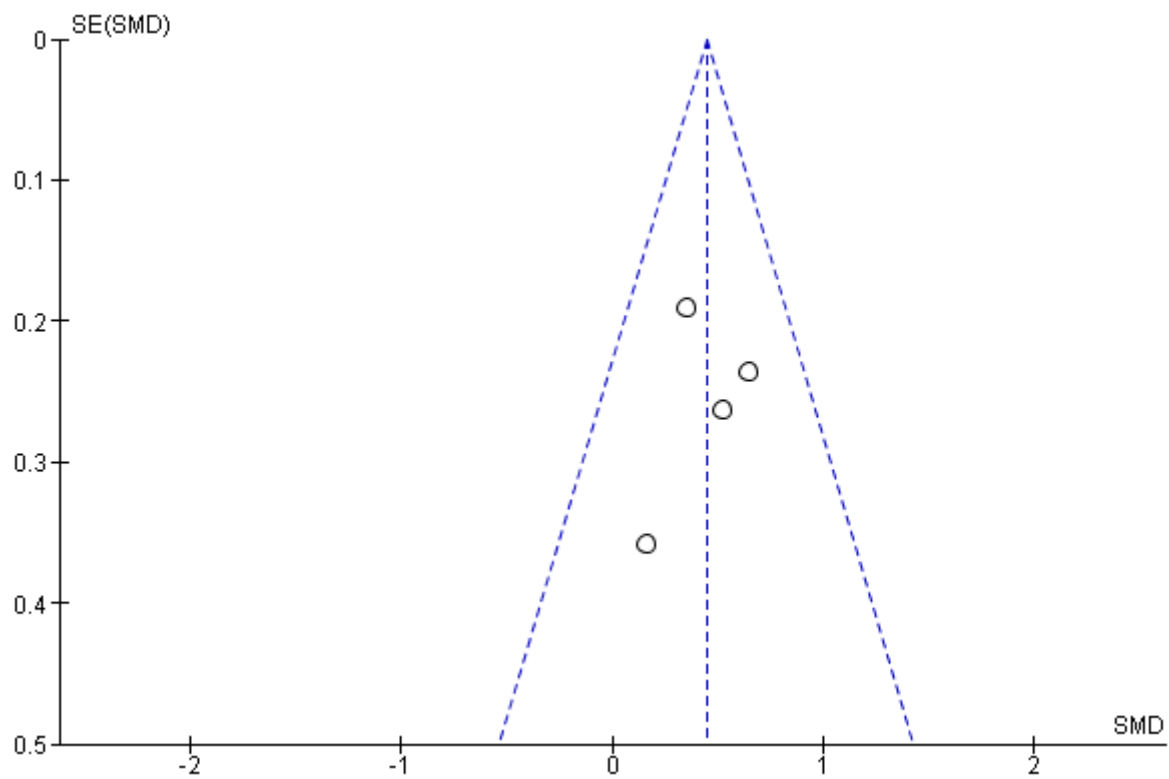
Supplementary Figure A.3. Funnel plot of the analysis involving the largest number of studies (Leptin: Microalbuminuria versus Normoalbuminuria).
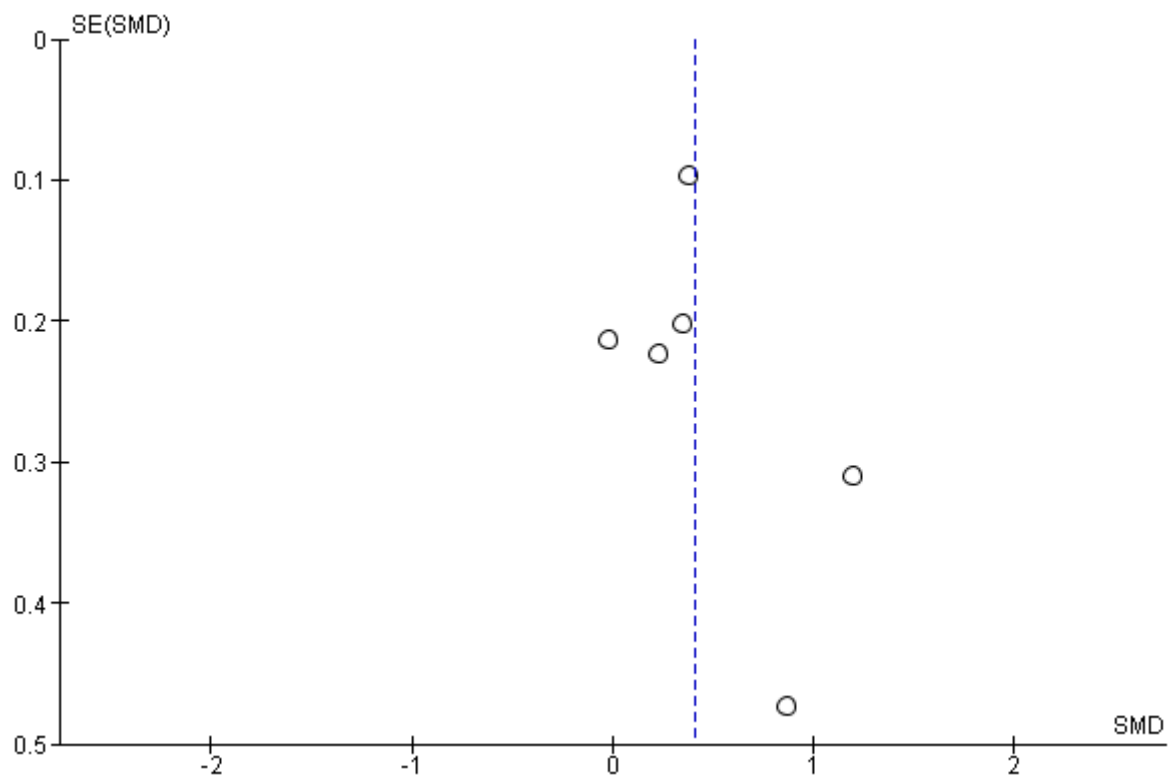
Supplementary Figure A.4. Funnel plot of the analysis Leptin: Neuropathy versus No Neuropathy.
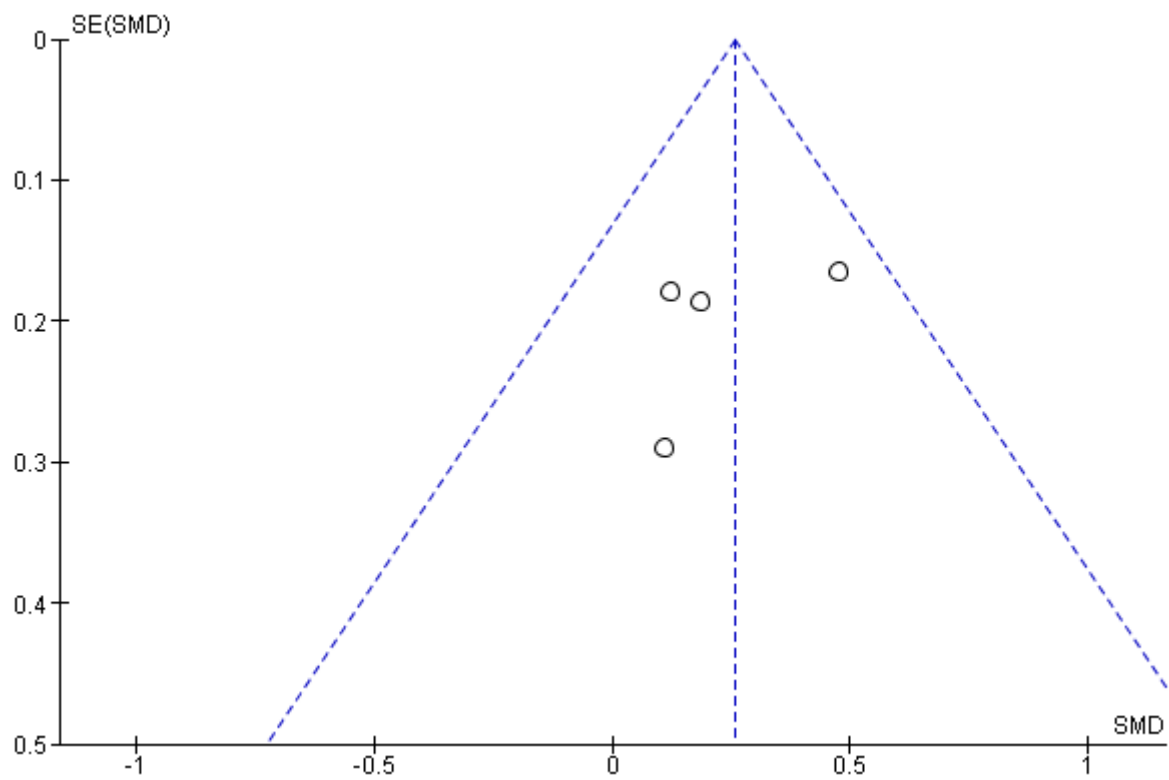
Supplementary Figure A.5. Funnel plot of the analysis Adiponectin: Retinopathy versus No Retinopathy.
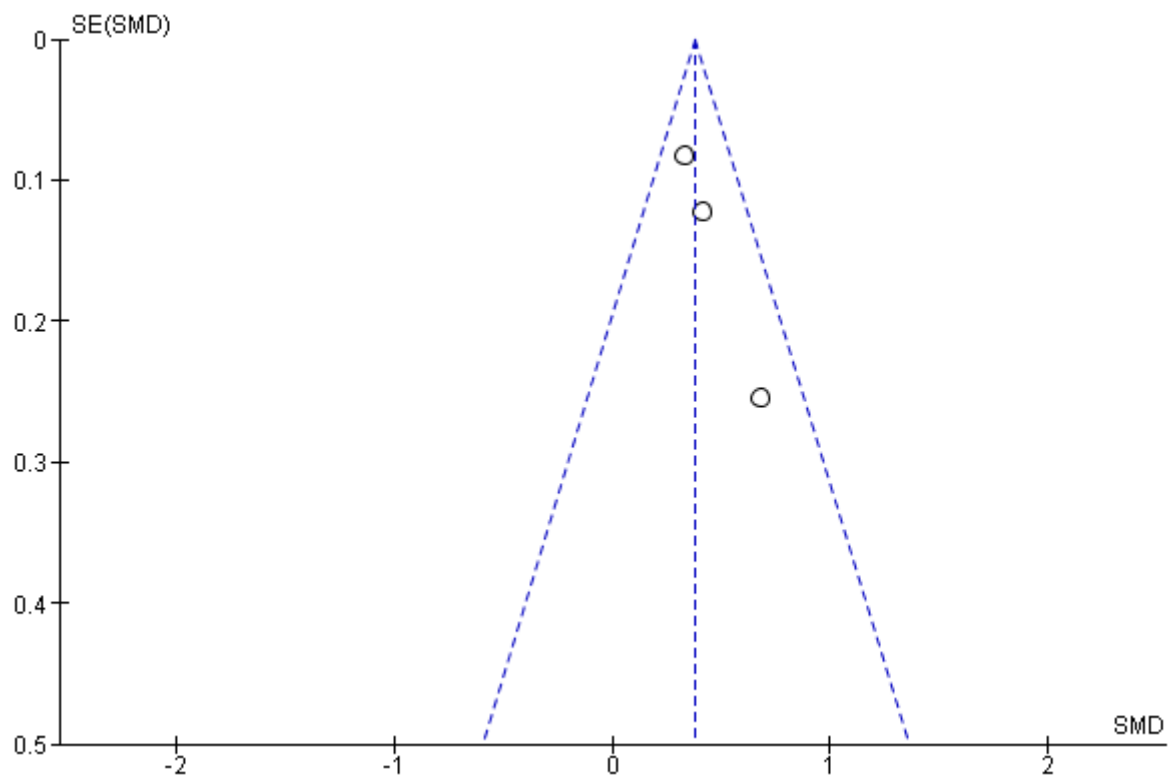
Supplementary Figure A.6. Funnel plot of the analysis Adiponectin: Microalbuminuria versus Normoalbuminuria.
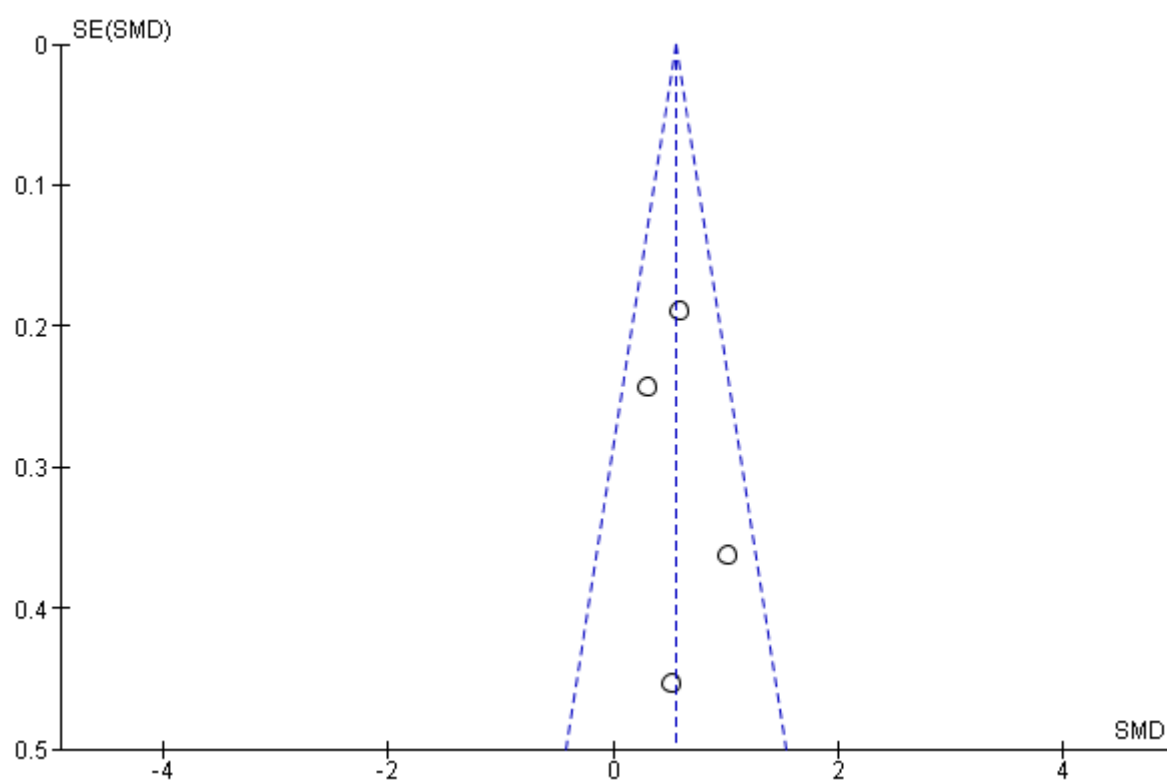
Supplementary Figure A.7. Funnel plot of the analysis Adiponectin: Macroalbuminuria versus Normoalbuminuria.
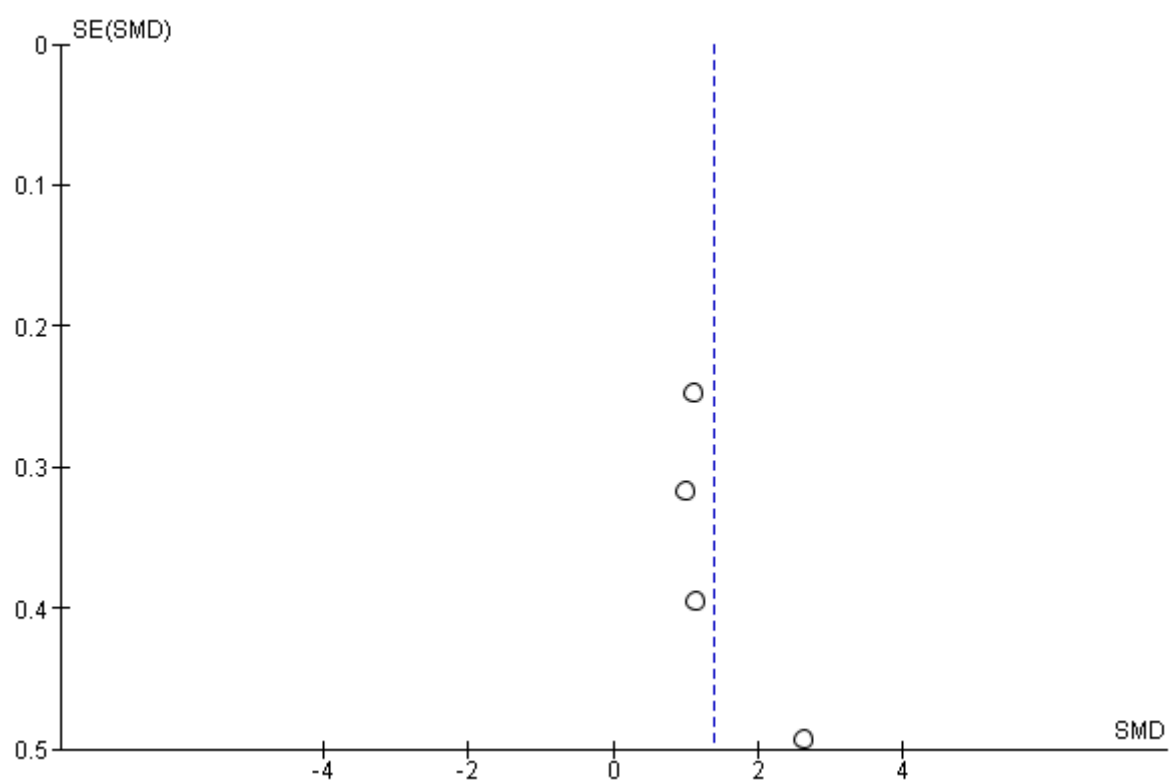
Supplementary Figure A.8. Funnel plot of the analysis Adiponectin: Macroalbuminuria versus Microalbuminuria.
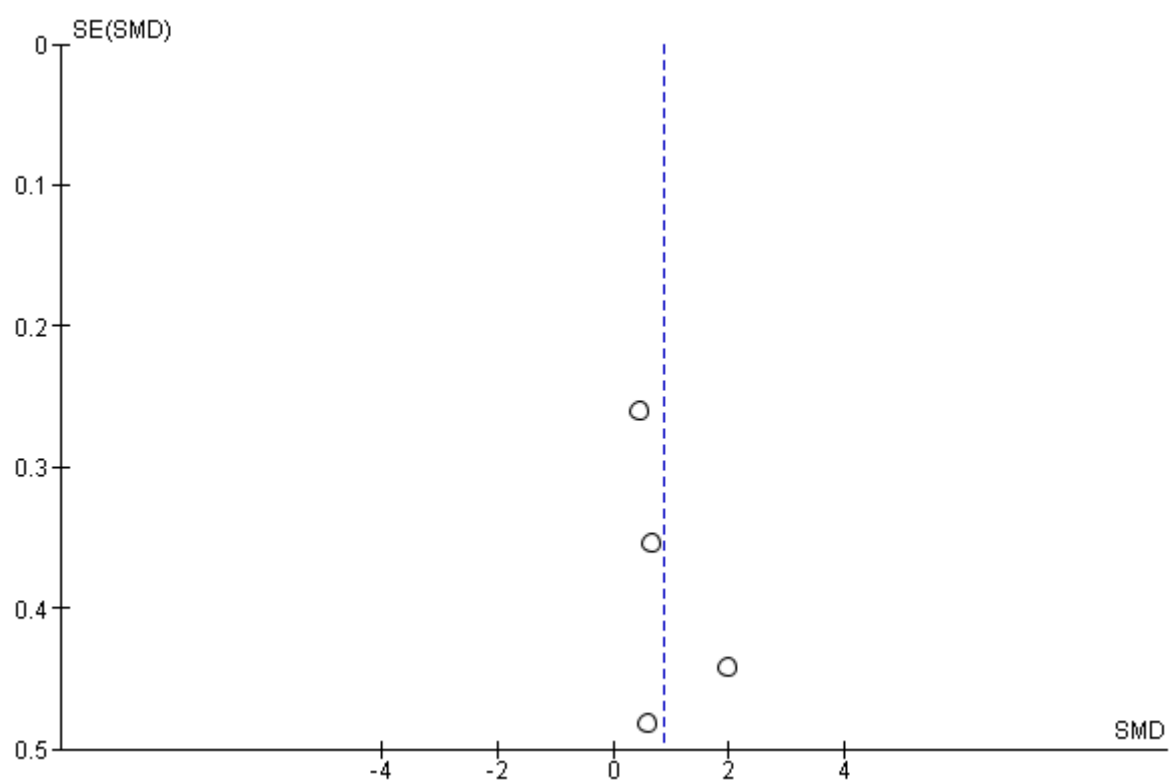
Supplementary Figure A.9. Funnel plot of the analysis Adiponectin: Neuropathy versus No neuropathy.
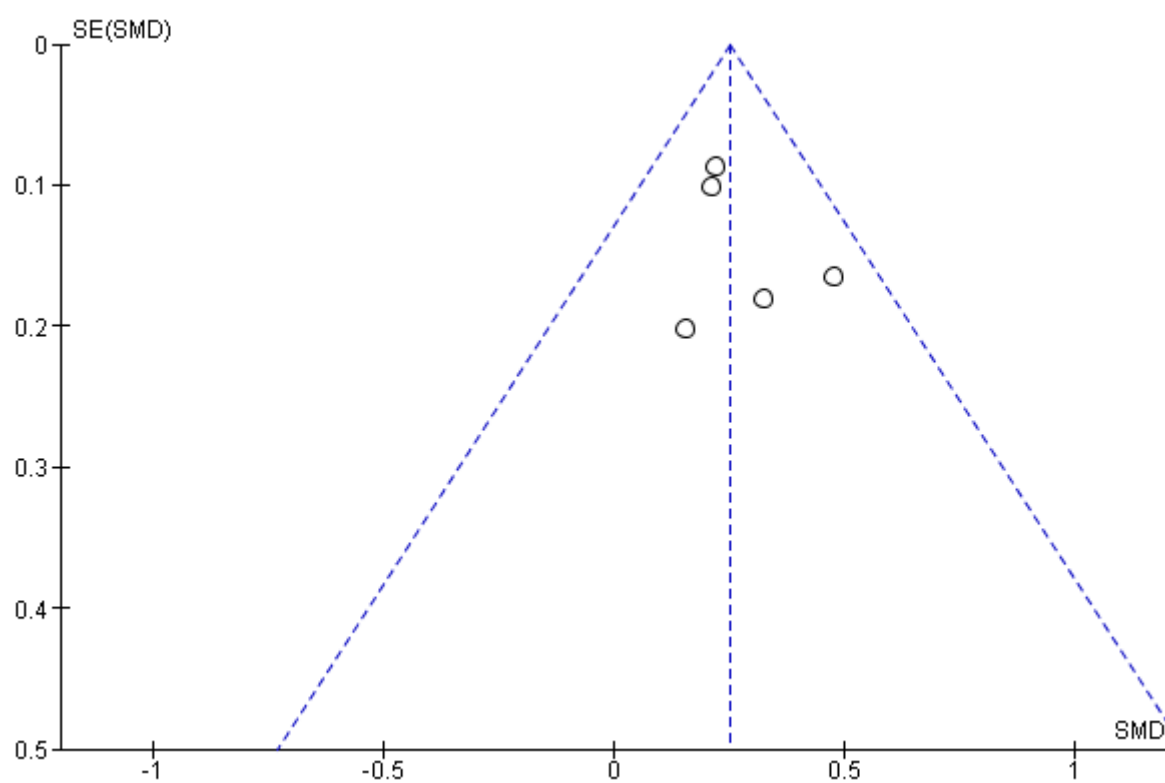
Supplementary Figure B. Leptin concentration (ng/mL) in patients with Diabetic Retinopathy versus No Diabetic Retinopathy.

1. Leptin Diabetic Retinopathy versus No Diabetic Retinopathy

Study or Subgroup	LEP DR Mean	SD	Total	LEP No DR Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Jung 2014	7.4	6.7	18	7.9	8.2	135	33.0%	-0.06 [-0.55, 0.43]
Sari 2010	28.5	20.5	37	26.7	17.1	120	34.6%	0.10 [-0.27, 0.47]
Uckaya 2000	13.42	6.12	37	5.8	3.7	33	32.4%	1.47 [0.94, 2.00]
Total (95% CI)			92			288	100.0%	0.49 [-0.38, 1.36]

Heterogeneity: Tau² = 0.54; Chi² = 21.45, df = 2 (P < 0.0001); I² = 91%
Test for overall effect: Z = 1.10 (P = 0.27)

Std. Mean Difference IV, Random, 95% CI — Higher levels in no DR   Higher levels in DR

2. Leptin Non-Proliferative Diabetic Retinopathy versus No Diabetic Retinopathy

Study or Subgroup	LEP Non PDR Mean	SD	Total	LEP No DR Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Sari 2010	27.4	18.3	25	26.7	17.1	120	51.2%	0.04 [-0.39, 0.47]
Uckaya 2000	11.5	3.5	20	5.8	3.7	33	48.8%	1.55 [0.91, 2.18]
Total (95% CI)			45			153	100.0%	0.78 [-0.70, 2.25]

Heterogeneity: Tau² = 1.06; Chi² = 14.86, df = 1 (P = 0.0001); I² = 93%
Test for overall effect: Z = 1.03 (P = 0.30)

Std. Mean Difference IV, Random, 95% CI — Higher levels in No DR   Higher levels in Non PDR

3. Leptin Proliferative Diabetic Retinopathy versus Non-Proliferative Diabetic Retinopathy

Study or Subgroup	LEP PDR Mean	SD	Total	LEP Non PDR Mean	SD	Total	Weight	Std. Mean Difference IV, Fixed, 95% CI
Sari 2010	30	19.9	12	27.4	18.3	25	48.3%	0.14 [-0.55, 0.82]
Uckaya 2000	16.1	9.2	17	11.5	3.5	20	51.7%	0.67 [0.00, 1.33]
Total (95% CI)			29			45	100.0%	0.41 [-0.07, 0.89]

Heterogeneity: Chi² = 1.19, df = 1 (P = 0.28); I² = 16%
Test for overall effect: Z = 1.68 (P = 0.09)

Std. Mean Difference IV, Fixed, 95% CI — Higher levels in Non PDR   Higher levels in PDR

4. Leptin Proliferative Retinopathy versus No Diabetic Retinopathy

Study or Subgroup	LEP PDR Mean	SD	Total	LEP No DR Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Sari 2010	30	19.9	12	26.7	17.1	120	50.6%	0.19 [-0.40, 0.78]
Uckaya 2000	16.1	9.2	17	5.8	3.7	33	49.4%	1.66 [0.98, 2.34]
Total (95% CI)			29			153	100.0%	0.91 [-0.53, 2.36]

Heterogeneity: Tau² = 0.98; Chi² = 10.25, df = 1 (P = 0.001); I² = 90%
Test for overall effect: Z = 1.24 (P = 0.21)

Std. Mean Difference IV, Random, 95% CI — Higher levels in No DR   Higher levels in PDR

Supplementary Figure C. Leptin concentration (ng/mL) in patients with Microalbuminuria versus Normoalbuminuria.

1. Meta-Analysis of six studies

	LEP Microalbuminuria			LEP Normoalbuminuria				Std. Mean Difference	Std. Mean Difference
Study or Subgroup	Mean	SD	Total	Mean	SD	Total	Weight	IV, Random, 95% CI	IV, Random, 95% CI
Cha 2012	32.6	11.2	41	29.7	14.1	40	17.2%	0.23 [-0.21, 0.66]	
Chung 2005	10.6	9.1	50	7.8	6.8	50	18.8%	0.35 [-0.05, 0.74]	
Fruehwald-Schultes 1999	21.16	18.3412	10	8.74	5.9767	10	6.6%	0.87 [-0.05, 1.80]	
Hanai 2010	6.9	4.4547	158	5.4	3.7719	344	27.4%	0.37 [0.18, 0.56]	
Kopiesy 2011	18.5	3.4	32	14.5	3.1	20	12.1%	1.20 [0.59, 1.81]	
Sari 2010	27.5	18.9	27	27.9	19.8	119	17.9%	-0.02 [-0.44, 0.40]	
Total (95% CI)			**318**			**583**	**100.0%**	**0.41 [0.14, 0.67]**	

Heterogeneity: Tau² = 0.06; Chi² = 12.03, df = 5 (P = 0.03); I² = 58%
Test for overall effect: Z = 2.96 (P = 0.003)

Higher levels in Normoalb   Hgher levels in Microalb

2. A leave-one-out sensitivity analysis excluding study Kopiesy et al.

	LEP Microalbuminuria			LEP Normoalbuminuria				Std. Mean Difference	Std. Mean Difference
Study or Subgroup	Mean	SD	Total	Mean	SD	Total	Weight	IV, Random, 95% CI	IV, Random, 95% CI
Cha 2012	32.6	11.2	41	29.7	14.1	40	13.3%	0.23 [-0.21, 0.66]	
Chung 2005	10.6	9.1	50	7.8	6.8	50	16.0%	0.35 [-0.05, 0.74]	
Fruehwald-Schultes 1999	21.16	18.3412	10	8.74	5.9767	10	3.1%	0.87 [-0.05, 1.80]	
Hanai 2010	6.9	4.4547	158	5.4	3.7719	344	53.3%	0.37 [0.18, 0.56]	
Kopiesy 2011	18.5	3.4	32	14.5	3.1	20	0.0%	1.20 [0.59, 1.81]	
Sari 2010	27.5	18.9	27	27.9	19.8	119	14.4%	-0.02 [-0.44, 0.40]	
Total (95% CI)			**286**			**563**	**100.0%**	**0.31 [0.14, 0.47]**	

Heterogeneity: Tau² = 0.00; Chi² = 4.42, df = 4 (P = 0.35); I² = 10%
Test for overall effect: Z = 3.66 (P = 0.0003)

Higher levels in Normoalb   Hgher levels in Microalb

Supplementary Figure D. Leptin concentration (ng/mL) in patients with Macroalbuminuria versus Normoalbuminuria.

1. Meta-Analysis of six studies

Study or Subgroup	LEP Macroalbuminuria Mean	SD	Total	LEP Normoalbuminuria Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Cha 2012	41.6	16.5	35	29.7	14.1	40	21.0%	0.77 [0.30, 1.24]
Chung 2005	15.8	17.9	63	7.8	6.8	50	23.7%	0.56 [0.18, 0.94]
Fruehwald-Schultes 1999	11.9	9.4236	10	4.13	2.9093	10	10.5%	1.07 [0.12, 2.02]
Kopiesy 2011	20.6	4.6	28	14.5	3.1	20	16.2%	1.48 [0.83, 2.13]
Sari 2010	30.9	23.3	11	27.9	19.8	119	17.0%	0.15 [-0.47, 0.77]
Wilson 1998	15.5	18.5921	10	14.1	17.1942	10	11.6%	0.07 [-0.80, 0.95]
Total (95% CI)			157			249	100.0%	**0.68 [0.30, 1.06]**

Heterogeneity: Tau² = 0.12; Chi² = 11.62, df = 5 (P = 0.04); I² = 57%
Test for overall effect: Z = 3.53 (P = 0.0004)

Higher levels in Normoalb   Higher levels in Macroalb

2. A leave-one-out sensitivity analysis excluding study Kopiesy et al.

Study or Subgroup	LEP Microalbuminuria Mean	SD	Total	LEP Normoalbuminuria Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Cha 2012	32.6	11.2	41	29.7	14.1	40	13.3%	0.23 [-0.21, 0.66]
Chung 2005	10.6	9.1	50	7.8	6.8	50	16.0%	0.35 [-0.05, 0.74]
Fruehwald-Schultes 1999	21.16	18.3412	10	8.74	5.9767	10	3.1%	0.87 [-0.05, 1.80]
Hanai 2010	6.9	4.4547	158	5.4	3.7719	344	53.3%	0.37 [0.18, 0.56]
Kopiesy 2011	18.5	3.4	32	14.5	3.1	20	0.0%	1.20 [0.59, 1.81]
Sari 2010	27.5	18.9	27	27.9	19.8	119	14.4%	-0.02 [-0.44, 0.40]
Total (95% CI)			286			563	100.0%	**0.31 [0.14, 0.47]**

Heterogeneity: Tau² = 0.00; Chi² = 4.42, df = 4 (P = 0.35); I² = 10%
Test for overall effect: Z = 3.66 (P = 0.0003)

Higher levels in Normoalb   Higher levels in Microalb

Supplementary Figure E. Leptin concentration (ng/mL) in patients with Macroalbuminuria versus Microalbuminuria.

Study or Subgroup	LEP Macroalbuminuria			LEP Microalbuminuria			Weight	Std. Mean Difference IV, Fixed, 95% CI
	Mean	SD	Total	Mean	SD	Total		
Cha 2012	41.6	16.5	35	32.6	11.2	41	26.5%	0.64 [0.18, 1.10]
Chung 2005	15.8	17.9	63	10.6	9.1	50	40.6%	0.35 [-0.02, 0.73]
Kopiesy 2011	20.6	4.6	28	18.5	3.4	32	21.3%	0.52 [0.00, 1.03]
Sari 2010	30.9	23.3	11	27.5	18.9	27	11.5%	0.16 [-0.54, 0.87]
Total (95% CI)			137			150	100.0%	0.44 [0.20, 0.68]

Heterogeneity: Chi² = 1.62, df = 3 (P = 0.66); I² = 0%
Test for overall effect: Z = 3.64 (P = 0.0003)

Std. Mean Difference
IV, Fixed, 95% CI

Higher levels in Microalb   Higher levels in Macroalb

Supplementary Figure F. Leptin concentration (ng/mL) in patients with Diabetic Neuropathy versus No Diabetic Neuropathy.

Study or Subgroup	LEP Diab Neuropathy			LEP No Diab Neuropathy			Weight	Std. Mean Difference IV, Fixed, 95% CI
	Mean	SD	Total	Mean	SD	Total		
Sari 2010	26.5	19.7	13	24.5	18.5	144	11.0%	0.11 [-0.46, 0.67]
Jung 2012	8.14	7.31	46	7.25	7.41	96	28.7%	0.12 [-0.23, 0.47]
Sari 2010	28.8	18.1	38	25.5	17.9	119	26.5%	0.18 [-0.18, 0.55]
Jung 2014	9.5	8.7	87	5.9	5.6	66	33.7%	0.48 [0.15, 0.80]
Total (95% CI)			**184**			**425**	**100.0%**	**0.26 [0.07, 0.44]**

Heterogeneity: Chi² = 2.76, df = 3 (P = 0.43); I² = 0%
Test for overall effect: Z = 2.66 (P = 0.008)

Std. Mean Difference
IV, Fixed, 95% CI

Higher levels in No DN   Higher levels in DN

Supplementary Figure G. Adiponectin concentration (µg/mL) in patients with Diabetic Retinopathy versus No Diabetic Retinopathy.

1. Adiponectin Diabetic Retinopathy versus No Diabetic Retinopathy
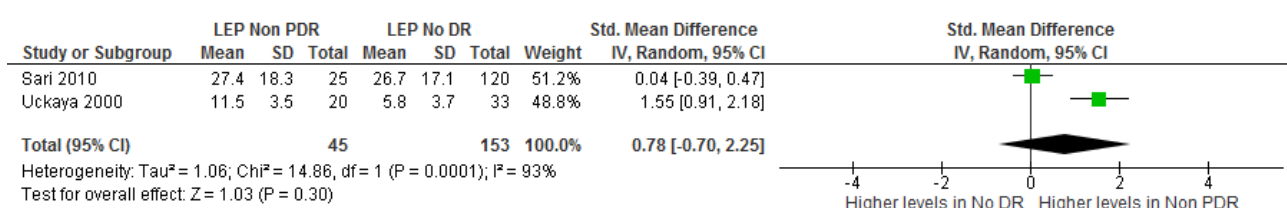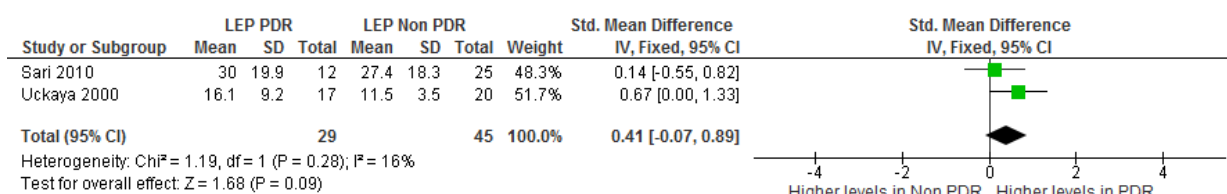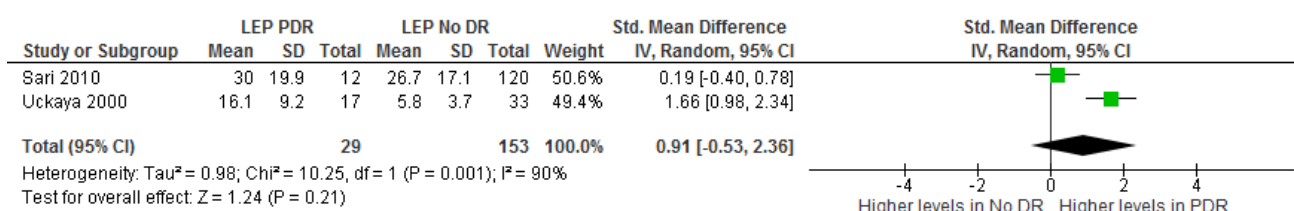
Study or Subgroup	ADP DR Mean	SD	Total	ADP No DR Mean	SD	Total	Weight	Std. Mean Difference IV, Fixed, 95% CI
Choe 2013	12.3	9.4	225	9.4	8.4	442	64.2%	0.33 [0.17, 0.49]
Jung 2014	5.5	4	18	3.3	3.1	135	6.8%	0.68 [0.18, 1.18]
Pradeepa 2015	8	4.8	81	6.4	3.6	406	29.1%	0.42 [0.18, 0.66]
Total (95% CI)			324			983	100.0%	**0.38 [0.25, 0.51]**

Heterogeneity: Chi² = 1.85, df = 2 (P = 0.40); I² = 0%
Test for overall effect: Z = 5.76 (P < 0.00001)

2. Adiponectin Non-Proliferative Diabetic Retinopathy versus No Diabetic Retinopathy

Study or Subgroup	ADP Non PDR Mean	SD	Total	ADP No DR Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Dossarps 2013	4.6	0	110	4	0	48		Not estimable
Kato 2008	8.3	4.3589	19	6.9	2.3812	63	50.4%	0.47 [-0.05, 0.99]
Pradeepa 2015	6.5	0	68	5.5	0	406		Not estimable
Yilmaz 2004	3.97	1.47	24	6.3	1.57	30	49.6%	-1.50 [-2.12, -0.89]
Total (95% CI)			221			547	100.0%	**-0.51 [-2.45, 1.43]**

Heterogeneity: Tau² = 1.87; Chi² = 23.29, df = 1 (P < 0.00001); I² = 96%
Test for overall effect: Z = 0.52 (P = 0.61)

3. Adiponectin Non-Proliferative Diabetic Retinopathy versus Proliferative Diabetic Retinopathy

Study or Subgroup	ADP Non PDR Mean	SD	Total	ADP Proliferative DR Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Kato 2008	8.3	4.3589	19	12.1	2.6944	6	46.3%	-0.91 [-1.86, 0.05]
Yilmaz 2004	3.97	1.47	24	3.16	1.83	20	53.7%	0.48 [-0.12, 1.09]
Total (95% CI)			43			26	100.0%	**-0.16 [-1.52, 1.20]**

Heterogeneity: Tau² = 0.80; Chi² = 5.80, df = 1 (P = 0.02); I² = 83%
Test for overall effect: Z = 0.23 (P = 0.82)

Supplementary Figure H. Adiponectin concentration (µg/mL) in patients with Microalbuminuria versus patients with Normoalbuminuria.

Study or Subgroup	ADP Microalbuminuria Mean	SD	Total	ADP Normoalbuminuria Mean	SD	Total	Weight	Std. Mean Difference IV, Fixed, 95% CI
Kato 2008	7.7	2.4495	24	7	2.3238	60	29.5%	0.29 [-0.18, 0.77]
Komaba 2006	10.8	7	44	7.2	5.6	86	48.7%	0.59 [0.22, 0.96]
Koshimura 2004	7.9	3.8	7	6.5	2.1	18	8.5%	0.51 [-0.38, 1.40]
Ran 2010	5.66	2.19	17	3.68	1.61	18	13.3%	1.01 [0.30, 1.72]
Total (95% CI)			92			182	100.0%	0.55 [0.29, 0.81]

Heterogeneity: Chi² = 2.78, df = 3 (P = 0.43); I² = 0%
Test for overall effect: Z = 4.17 (P < 0.0001)

Std. Mean Difference IV, Fixed, 95% CI

Higher Levels in Normoalb   Higher Levels in Microalb

Supplementary Figure I. Adiponectin concentration (μg/mL) in patients with Macroalbuminuria versus patients with Normoalbuminuria.

1. Meta-Analysis of four studies

Study or Subgroup	ADP Macroalbuminuria			ADP Normoalbuminuria			Weight	Std. Mean Difference IV, Random, 95% CI	Std. Mean Difference IV, Random, 95% CI
	Mean	SD	Total	Mean	SD	Total			
Kato 2008	9.5	3.245	13	7	2.3238	60	27.0%	0.99 [0.37, 1.61]	
Komaba 2006	14.3	8.7	23	7.2	5.6	86	30.6%	1.11 [0.62, 1.59]	
Koshimura 2004	11	5.5	13	6.5	2.1	18	23.2%	1.13 [0.35, 1.90]	
Ran 2010	13.28	4.98	15	3.68	1.61	18	19.1%	2.64 [1.67, 3.60]	
Total (95% CI)			**64**			**182**	**100.0%**	**1.37 [0.78, 1.97]**	

Heterogeneity: Tau² = 0.24; Chi² = 9.06, df = 3 (P = 0.03); I² = 67%
Test for overall effect: Z = 4.53 (P < 0.00001)

Higher levels in Normoalb   Higher levels in Macroalb

2. A leave-one-out sensitivity analysis excluding study Ran et al.

Study or Subgroup	ADP Macroalbuminuria			ADP Normoalbuminuria			Weight	Std. Mean Difference IV, Random, 95% CI	Std. Mean Difference IV, Random, 95% CI
	Mean	SD	Total	Mean	SD	Total			
Kato 2008	9.5	3.245	13	7	2.3238	60	30.3%	0.99 [0.37, 1.61]	
Komaba 2006	14.3	8.7	23	7.2	5.6	86	50.1%	1.11 [0.62, 1.59]	
Koshimura 2004	11	5.5	13	6.5	2.1	18	19.6%	1.13 [0.35, 1.90]	
Ran 2010	13.28	4.98	15	3.68	1.61	18	0.0%	2.64 [1.67, 3.60]	
Total (95% CI)			**49**			**164**	**100.0%**	**1.08 [0.73, 1.42]**	

Heterogeneity: Tau² = 0.00; Chi² = 0.11, df = 2 (P = 0.95); I² = 0%
Test for overall effect: Z = 6.16 (P < 0.00001)

Higher levels in Normoalb   Higher levels in Macroalb

Supplementary Figure J. Adiponectin concentration (μg/mL) in patients with Macroalbuminuria versus patients with Microalbuminuria.

1. Meta-Analysis of four studies

2. A leave-one-out sensitivity analysis excluding study Ran et al.

Supplementary Figure K. Adiponectin concentration (µg/mL) in patients with Diabetic Neuropathy versus No Diabetic Neuropathy.
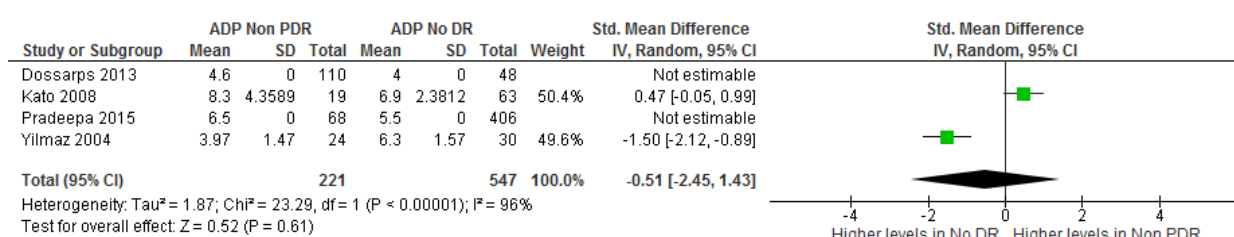
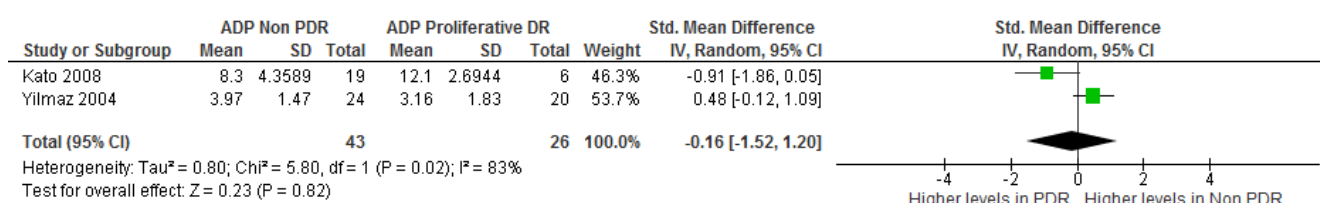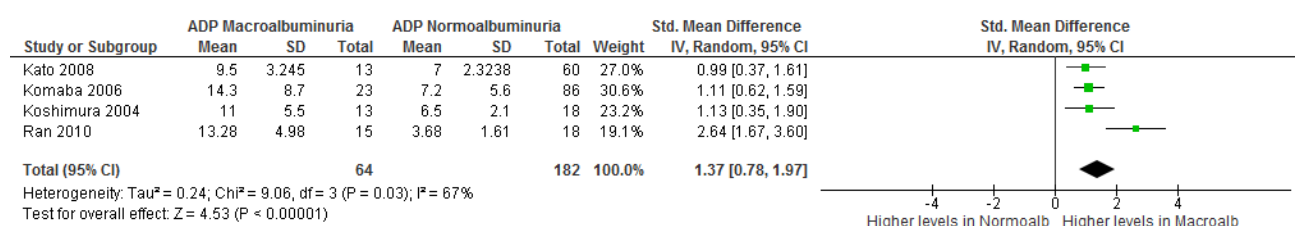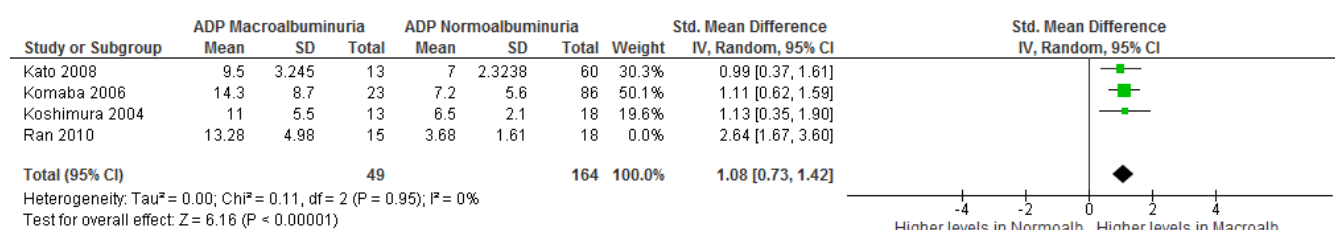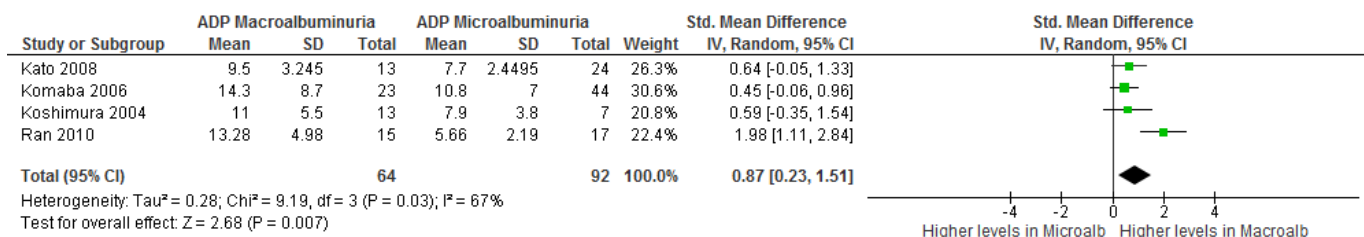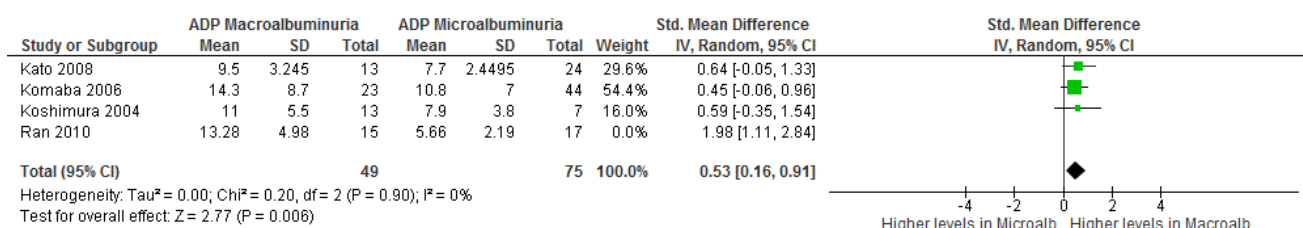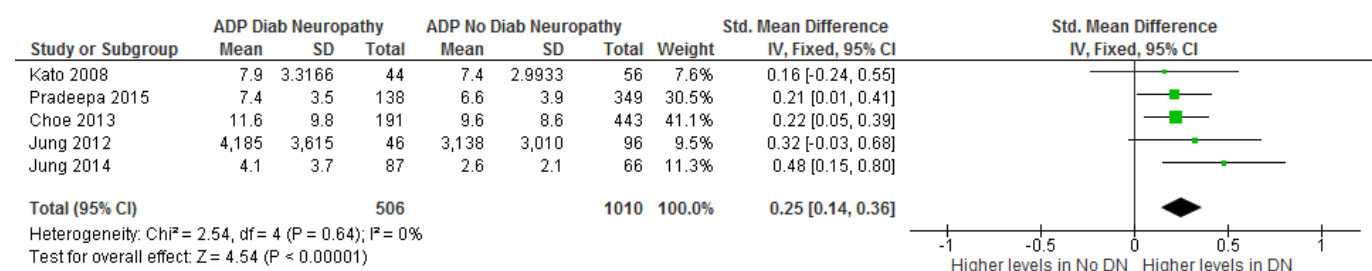| Study or Subgroup	ADP Diab Neuropathy Mean	SD	Total	ADP No Diab Neuropathy Mean	SD	Total	Weight	Std. Mean Difference IV, Fixed, 95% CI
Kato 2008	7.9	3.3166	44	7.4	2.9933	56	7.6%	0.16 [-0.24, 0.55]
Pradeepa 2015	7.4	3.5	138	6.6	3.9	349	30.5%	0.21 [0.01, 0.41]
Choe 2013	11.6	9.8	191	9.6	8.6	443	41.1%	0.22 [0.05, 0.39]
Jung 2012	4,185	3,615	46	3,138	3,010	96	9.5%	0.32 [-0.03, 0.68]
Jung 2014	4.1	3.7	87	2.6	2.1	66	11.3%	0.48 [0.15, 0.80]
Total (95% CI)			**506**			**1010**	**100.0%**	**0.25 [0.14, 0.36]**

Heterogeneity: Chi² = 2.54, df = 4 (P = 0.64); I² = 0%
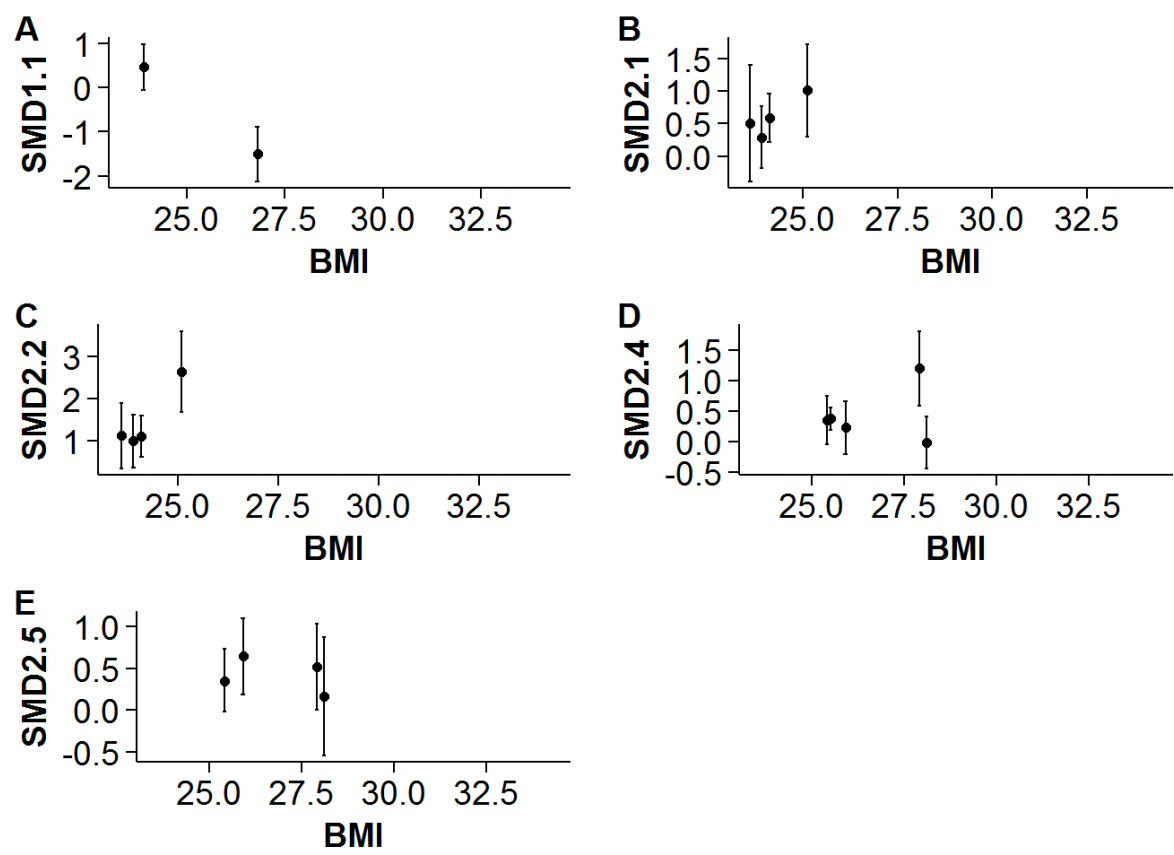Test for overall effect: Z = 4.54 (P < 0.00001)

Supplementary Figure L. Meta-regression of the impact of age on effect size [A= adiponectin in non PDR vs. No DR; B= adiponectin in micro vs normo; C= adiponectin in macro vs normo; D= leptin in micro vs normo; E= leptin in macro vs normo].
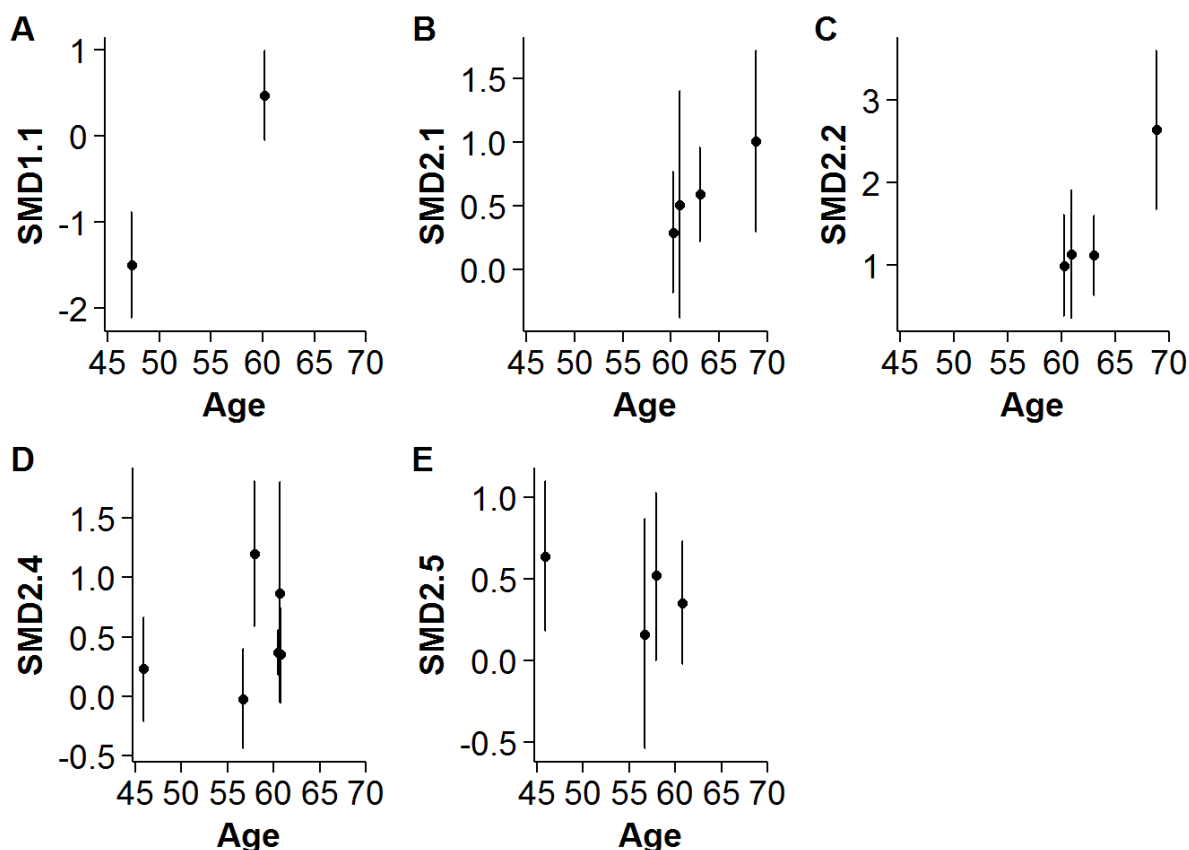
Note: In meta-regression, the effect size (SMD) is plotted on the vertical axis and the study-level co-variate is plotted on the horizontal axis. As in a simple linear regression, trends are investigated to assess whether there is linearity between the two variables, more specifically if there is dependency of one variable on the other (for example increasing blood pressure with age). In this instance, we are investigating whether the difference between leptin and adiponectin in case and control groups may be explained by age. Plots B and C show a small weak positive trend suggesting that age may explain some of the difference in adiponectin between case and control groups.

Supplementary Figure M. Meta-regression of the impact of BMI on effect size [A= adiponectin in non PDR vs No DR; B= adiponectin in micro vs normo; C= adiponectin in macro vs normo; D= leptin in micro vs normo; E= leptin in macro vs normo].

Supplementary Figure N. Meta-regression of the impact of the duration of diabetes on effect size [A= adiponectin in non PDR vs NO DR; B= adiponectin in micro vs normo; C= adiponectin macro vs normo; D= leptin in micro vs normo; E= leptin in macro vs normo].