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Cardiovascular effects of calcium supplements

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A thesis submitted in fulfilment of the requirement for the degree of Doctor of Philosophy in Medicine, the University of Auckland, 2019.

Abstract

Aim

In view of the association of calcium supplements with myocardial infarction and stroke, we sought to understand whether this effect persisted after their discontinuation and whether this risk varied across different patient subgroups. We also sought to understand what effect calcium supplements might exert in a mouse model of accentuated inflammation.

Method

We continued follow-up of the Auckland Calcium study participants for a further 5 years to understand the persistence of the harmful effects of calcium supplements in the context of bone protective actions. We re-analysed the Women's Health Initiative Calcium and Vitamin D (WHI CaD) study and performed similar analyses in our pooled, patient-level meta-analysis dataset to detect if calcium supplements conferred a differential cardiovascular risk across various patient groups.

Finally, we investigated whether calcium supplements increased vascular calcification, altered the serum mineral profile and produced changes in gene expression of vascular cells in a mouse model of accentuated inflammation. We were also interested as to whether providing calcium over 24 hours would mitigate any negative effects of a single calcium bolus.

Findings

Post-trial follow-up of the Auckland Calcium study showed that allocation to calcium reduced forearm and vertebral fractures over the entire follow up period, but did not influence the risk for myocardial infarction, stroke, transient ischaemic attacks, or death. In the bone

mineral density sub-study, there were no between-group differences in the percentage change from baseline at any site, at 10 years.

Results from the meta-analysis of calcium monotherapy and the re-analysis of the WHI CaD were strikingly similar in terms of risk for myocardial infarction and stroke, however, we found no evidence for an interaction between allocation to calcium and important baseline characteristics.

In adiponectin knockout mice, a once daily bolus dose of calcium altered serum mineral profile and genes implicated in vascular calcification.

Conclusion

The harmful effects of calcium supplements do not differ across differing patient subgroups and do not persist once discontinued. At the cell level, calcium supplements may play a role in the phenotypic change of vascular smooth muscle cells promoting vascular disease, but this will need to be confirmed in targeted experiments.

In memory of my parents,

Ta'atele Wigg 02/02/1956 - 9/10/2010

and

Desmond Wigg 05/03/1953 - 31/10/2016

"Now to Him who is able to keep you from stumbling,

And to present you faultless before the presence of His glory with exceeding joy,

To God our Saviour, who alone is wise,

Be glory and majesty,

Dominion and power,

Both now and forever.

Amen."

Jude 24-25

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Certification by Co-Authors

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1. INTRODUCTION

1.1. Osteoporosis: an increasing global concern.

Osteoporosis is a disease of the skeleton characterized by low bone strength leading to bone fragility and susceptibility to fracture (1). The term was coined in the 19th century (from Greek: οστούν/ ostoun meaning "bone" and πορος/poros meaning "pore") by French pathologist Jean Georges Chretien Frederic Martin Lobstein who observed that some patients' bones had larger holes than normal (2). Bone strength is the combination of bone mineral density as measured by dual-energy x-ray absorptiometry, and bone quality encompassing bone microarchitecture, turnover, mineralization and microdamage accumulation. Although osteoporotic fractures may occur in the young, they predominantly result from the ageing process substantiated by observations that ~50% of white women over the age of 50 years (and ~20% of males) will sustain a fragility fracture in their remaining lifetime (3).

Osteoporosis and more specifically its clinical manifestation – fragility fracture, are a major health burden both to the individual and to the global health economy. In recent years many groups have analysed the current and projected costs to local and global economy with staggering results as life expectancies increase and the number of at risk individuals' increase (4-10). In the United States the annual costs for osteoporosis-related fractures were estimated to be US $16.9 billion in 2005, a figure expected to rise to $US25.3 billion by the year 2025 (4). This figure was €37 billion for the 27 countries in the European Union in 2010 with a 25% increase in costs expected in 2025 (8). Brown *et al*. estimated that in New Zealand more than 80,000 people would suffer an osteoporotic fracture in 2007 for a cost of $330 million (11). Approximately two-thirds of these fractures occurred in women. They projected that there would be a 37% increase in fractures with costs increasing to $458 million by the year

2020. However, with increasing life expectancies, and the proportion of the population aged 65 and over projected to increase from 15% in 2016 to 20% in 2028, we can expect the magnitude of the problem to be much larger than initially predicted (12,13).

Although fragility fractures can occur in all bones, they most commonly occur in the hip, spine, and wrist. Hip fractures are of particular concern. Not only do they result in increased mortality of ~25% within the first year, they are also associated with on-going morbidity culminating in short and/or long-term rest home care as well as a significant cost burden to the health care system as a result of acute and long-term care (14-20). Unsurprisingly, they account for the highest disability adjusted life years lost due to osteoporosis (18,21,22). Burge *et al.* estimated that hip fractures accounted for 14% of all incident fractures but 72% of total fracture cost (4). Melton *et al.* estimated the lifetime risk for hip fractures to be 17.5% in white women and 6.0% in white men (23).

Vertebral fractures, like hip fractures, are associated with an increase in mortality but of delayed onset (24). The increased risk remains after adjusting for chronic medical conditions (25) and increases further with number of previous vertebral fractures sustained. Although vertebral fractures do not usually lead to long term nursing home care, they are associated with significant morbidity in the form of pain and deformity, the latter negatively impacting pulmonary function (25-27). Having sustained one osteoporotic fracture, patients are at an increased risk for developing other types of fractures, and women have a 20% chance of a further vertebral fracture within 1 year of an incident vertebral fracture (28). This first incident provides an opportunity for clinicians to instigate preventative strategies protecting against further fractures. Unfortunately, many vertebral fractures are subclinical in nature, with approximately two-thirds of vertebral fractures avoiding diagnosis at the time of occurrence (6,29,30). This means that many individuals at high risk for a further fragility

fracture are not brought to medical attention until sustaining a second fracture at which time the associated morbidity and risk of successive fracture is further increased.

Distal forearm fracture incidence increases rapidly after menopause but reaches a plateau by about age 65 years (6). It shows a strong female to male preponderance of 4:1 (22) with no associated increase in mortality (31). Patients with distal forearm fractures rarely require nursing home care and largely remain functionally independent in contrast to those suffering from either hip or spine fracture (5).

Jacobzone predicted that in 2013 we would be living in a time of increased dependency ratio owing to the baby boom generation who are now aged over 65 years as well as gains in life expectancy (32). As a result, the impact of osteoporosis continues to grow, increasing the pressure for accurate identification of at-risk individuals and effective prevention and treatment strategies. A further consideration regarding the future impact of the disease is the importance of exercise and bone loading to attain peak bone mass. Peak bone mass accrual velocity occurs at 12.5 years in females and 14.1 years in males (33). As our current younger generations now grow in a time of computer games and disappearing back yards it will be interesting to see whether osteoporosis and fragility fractures emerge at a younger age in those who theoretically, will not have achieved an appropriate peak bone mass.

1.1.1. Menopause increases the risk of osteoporosis in women.

After peak bone mass accrual velocity during puberty, females continue to increase bone mass to age 18 years with a decrease in rate of gain thereafter. Depending on site and other environmental inputs, gains or losses may be made to the skeleton prior to menopause, although bone accrual post aged 30 years is small (34-38). Whether bone is gained or lost prior to menopause has no influence on the striking rate of bone loss that occurs post-menopause which may be as much as 5% per year (39-41). This accelerated period of bone

loss continues for at least two years then bone loss continues thereafter at a slower rate (38,42). In the vertebrae this acceleration in bone loss amounts to nearly 6% of attained peak vertebral mass each year, resulting in an overall reduction in vertebral bone mineral content of 23% in postmenopausal women without osteoporosis and 41% in women with osteoporosis (40). In his literature review, Mazess found that the cumulative actions of oestrogen loss at menopause, in conjunction with age-related losses, translated to a 15-20% loss of compact bone and a 30% loss of trabecular bone at age 60 years (38). It is generally agreed that bone loss occurs as a result of increasing age but, in females, is accelerated for a time during menopause through the loss of oestrogen (38).

Osteoporosis is attributed to the accelerated bone loss that occurs secondary to the reduction of oestrogen at menopause (38,43-45). The observation that bone loss occurs prior to menopause, along with the weak association of oestrogen levels with the rate of postmenopausal bone loss (46), highlight the multiple influences and the complex interplay of events at this time.

1.1.2. Treatment.

Adopting multimodal prevention strategies complements the effectiveness of pharmacological treatments in the prevention of osteoporotic fractures. These strategies are comprised of both population-based interventions such as a healthy diet, weight-bearing exercises, and lifestyle modifications such as the cessation of cigarette smoking and moderation of alcohol intake; and also tailored to the individual such as limiting falls risks by home assessments, limiting the impact of a fall by the appropriate use of hip protectors, and optimisation of comorbidities and other health factors that may contribute to an increased risk for falls such as poor eyesight, reduced hearing and inadequate glycaemic control in individuals with diabetes mellitus (47).

1.1.2.1. Pharmacological treatment.

Calcium and Vitamin D supplements are beneficial for those individuals who have low circulating Vitamin D_3 levels in an institutional setting (48). However, they are not effective in fracture reduction in community-based healthy post-menopausal women (49). (Please see section 1.5 Calcium and the prevention of osteoporotic fractures)

Bisphosphonates are antiresorptive agents that work by inhibiting osteoclast function and by reducing the activation frequency of new bone modelling units (50). They may also increase survival of osteoblasts and osteocytes (51). They are inorganic pyrophosphate analogues that adsorb to hydroxyapatite with high affinity, preferentially into sites of active bone remodelling (52). Unlike the first generation bisphosphonates, the second and third generation bisphosphonates have nitrogen-containing side arms which increases their affinity for hydroxyapatite (52). Alendronate (second generation) (53,54), risedronate (55) and zoledronate (third generation) (56) reduce vertebral fractures and hip fracture incidence (53,56,57). What is unclear is what duration of treatment is required for long-term fracture prevention and whether drug-free holidays are appropriate (50). Recent evidence suggests that yearly dosing with zoledronate may be unnecessary (57), and others have shown persisting benefits on bone mineral density 5 years after a single dose (58). In their systematic review, Eriksen *et al.* found that 3 to 5 years of treatment with alendronate or zoledronate conferred continued anti-fracture benefit for 3 to 5 years after treatment was discontinued (59). Alendronate shows a persisting effect on the prevention of non-vertebral fractures beyond 5 years of treatment, with patients randomised to a further 5 years of alendronate showing no decrease in fracture risk compared with those who discontinued alendronate after 5 years (60).

Raloxifene is a selective oestrogen receptor modulator that effectively reduces vertebral fractures as shown in the MORE (multiple outcomes of raloxifene evaluation) study, but not nonvertebral fractures (61,62). It is, however, associated with adverse cardiovascular side effects. In a large study comprised of >10,000 postmenopausal women, raloxifene was associated with an increased risk for fatal stroke (hazard ratio, 1.49; 95% confidence interval, 1.00 to 2.24) and venous thromboembolism (hazard ratio, 1.44; 95% confidence interval, 1.06 to 1.95) (63).

Denosumab is a human monoclonal antibody that binds to RANK-ligand (receptor activator of nuclear kappa-B ligand) and thereby reversibly prevents osteoclast differentiation and bone resorption. In the large international FREEDOM study, denosumab prevented vertebral (hazard ratio 0.32; 95% confidence interval 0.26 to 0.41), hip (hazard ratio 0.60; 95% confidence interval 0.37 to 0.97; $p = 0.04$) and non-vertebral fractures (hazard ratio 0.80; 95% confidence interval 0.67 to 0.95; $p = 0.01$) after three years of intervention (64). An emerging concern is the phenomenon of rebound-associated vertebral fracture after treatment is discontinued. Nine women were recently reported with this phenomenon, of which seven were osteoporotic (65). These case reports are striking both in the number of fractures that occurred (50 fractures in 9 women) and the short time frame within which they occurred, between 9 and 16 months after the last denosumab injection. Anastasilakis *et al.* identify increasing osteoclast formation and activity, attributable in part to the reduction in the non-coding RNAs miR-503 and miR-222-2 that downregulate osteoclastogenesis, as a possible mechanism for this phenomenon (66).

Teriparatide is the recombinant form of the bioactive portion of the human parathyroid hormone. Although it increases the activation frequency of bone remodelling units, because it also promotes osteoblast survival and increases differentiation of osteoblast precursors, the

net effect is increased trabecular bone. Teriparatide is also active in cortical bone where it induces periosteal apposition and endosteal resorption, however, gains in cortical bone mineral density are not observed (67). Administered as a once-weekly injection, teriparatide reduces the incidence of vertebral, non-vertebral and hip fractures (68,69). Adherence and persistence with treatment impacts the observed fracture risk reduction with increased adherence and persistence associated with greater risk reduction (68).

Together, these pharmacological treatment options present the possibility of managing the upward spiral of osteoporotic fractures worldwide. At least two challenges, however, lie ahead. Firstly, individuals who have sustained a previous fragility fracture continue to be at a higher risk for further fracture in spite of the initiation of effective treatment (70). Secondly, researchers in the United States have found a decrease in the number of patients taking fracture-preventing medicines, a finding that is likely multifactorial pertaining to both clinician and the individual. Therefore, we must continue to better equip our armamentarium and relentlessly pursue and minimize factors which may inflict harm as a result of our treatment.

"First do no harm." Adaptation of the Hippocratic Oath.

1.2. Osteoporosis is associated with cardiovascular disease.

In New Zealand females greatly outnumber males in osteoporotic hospital admissions from ages 55-59 years with or without a pathological fracture. For cerebrovascular disease and cardiovascular disease (CVD) this sex preponderance is not observed until many years later at age 85 years (71). Although the comparison between the sex differences in prevalence between osteoporosis and CVD does not suggest a common thread, there is evidence implying otherwise.

Osteoporosis is associated with an increased risk of CVD. In a meta-analysis of over 1.1 million participants contained in 28 studies of which 64.4% were women, low bone mineral density was associated with an increased risk of developing coronary artery disease, cerebrovascular disease and CVD-associated death (72). Fracture presence at baseline also increased the risk for an incident CVD event with a hip fracture conferring a higher risk (hazard ratio 1.48; 95% confidence interval 1.22, 1.80; $p < 0.0001$) than vertebral fractures (hazard ratio 1.26; 95% confidence interval, 1.02, 1.55; $p = 0.04$). This was supported in a further meta-analysis: postmenopausal women with osteoporosis had an odds ratio for the incidence of atherosclerotic vascular abnormalities of 2.05 (95% confidence interval: 1.13, 3.72; $p = 0.02$) compared with women who did not have osteoporosis (73) which was further accentuated when compared with women who had normal bone mineral density (odds ratio 4.29, 95% confidence interval 2.37, 7.77; $p < 0.00001$).

Menopause increases the risk of osteoporosis and through its effect on many cardiovascular risk factors also increases CVD risk. This is supported by the finding that women with early menopause have an elevated risk of coronary heart disease and ischaemic stroke (74-76).

1.2.1. Influence of menopause on cardiovascular disease.

The PREDICT study was a large prospective cohort study of primary care patients in New Zealand undergoing CVD risk assessment (77). Given previous CVD risk assessment tools were based on data derived from a United States cohort in the 1960s and 1970s this study provides risk predictions that are validated for New Zealanders. Although the risk equations are not yet available, recommendations in the 2018 CVD risk assessment consensus statement can be applied now (78). Menopause cannot be responsible for risk factors such as age, family history (of CVD and hypercholesterolaemia), ethnicity, or personal decisions (smoking), but oestrogen withdrawal has an influence on many of the remaining risk factors.

1.2.1.1. Systolic blood pressure.

Before menopause, women have a lower incidence of hypertension compared with males. At menopause this sex difference in prevalence changes such that by age 60 years, females have higher rates of systolic hypertension (79-82). Given that this is not a consistent finding, and studies of oestrogen therapy have failed to show a beneficial effect and may even increase CVD risk (83), many have questioned the role of sex hormones in the pathogenesis of hypertension. There is however, a plethora of biological evidence to support oestrogen withdrawal in the pathogenesis of postmenopausal hypertension, and the conflicting findings after oestrogen therapy may be a result of the complex interaction with oestrogen and its receptors.

In the endothelium, oestrogen is able to signal through the oestrogen receptors ERα and ERβ, although ERα appears to mediate the beneficial vascular effects. These receptors are present both on the endothelial surface, mediating non-genomic actions, and at the nucleus inducing their genomic effects. A G-protein coupled receptor GPCR30 also mediates oestrogen functions in the nucleus (84). Cell signalling can involve multiple points of contact with each

cell and therefore the oestrogen-induced phenotype depends on the relative abundance of these three receptors and the interplay of their downstream effects. Additionally, a diversity in response can be induced by alternative RNA splicing leading to multiple receptor isoforms and epigenetic changes (85). Of interest is the finding that the total and relative expression of ERα and ERβ in the urogenital tissue of female CD-1 mice differs after bilateral oophorectomy with and without supplemental oestrogen (86). This change in the relative abundance of receptors alters the oestrogen-induced phenotype of these tissues post-menopause. There is some evidence to suggest that oestrogen itself can regulate receptor abundance (85). An upregulation of ERβ has been identified in human uterine arteries according to the number of years since menopause (87). If a post-menopausal change in the relative abundance of oestrogen receptors is confirmed in vascular cells, this may contribute to the disappointing findings of pharmacological studies and also explain the 'window effect' where oestrogen therapy has beneficial effects if administered early in the perimenopausal transition, but deleterious when taken by women significantly past menopause (88-90).

Oestrogen modulates blood pressure through its effects on endothelial function, renin-angiotensin system, arterial stiffness and oxidative stress (91). Oestrogen is known to increase endothelial nitric oxide synthase through both non-genomic and genomic pathways, with increases in nitric oxide and decreases in the vasoconstrictor factor endothelin-1 inducing vasodilation (84,85). The loss of nitric oxide post-menopause mediates in part the increased vulnerability for Na^+-induced hypertension (92-94).

Oestrogen modulates the renin-angiotensin-aldosterone system (RAAS) by altering the gene expression of pertinent enzymes and receptors. Angiotensin II is a potent vasoconstrictor both stimulating vasoconstriction in blood vessels and stimulating aldosterone release from the adrenal gland, thereby increasing sodium reabsorption from the kidneys. It acts through its receptors angiotensin receptor subtype 1 (AT_1) and subtype 2 (AT_2). Angiotensin II is

produced catalytically in a two-step process. Angiotensinogen released from the liver is cleaved by renal renin to form angiotensin I which undergoes further cleavage by the enzyme angiotensin-converting enzyme (ACE), releasing the active angiotensin II. In apolipoprotein knockout mice, oestrogen, through ERα receptors, decreases kidney expression of the enzymes ACE and its homologue ACE_2, and the angiotensin II receptor AT_1, while inducing a profound increase in AT_2 receptors (95). Conversely, the AT_2 receptor has effects that oppose AT_1 receptor stimulation. In the kidney, AT_2 increases bradykinin and nitric oxide end products which contribute to normalizing systolic blood pressure (96). This accounts for the seemingly paradoxical effect of oestrogen on the expression of the two angiotensin receptor subtypes as appreciated in the aforementioned study.

Decreases in oestrogen are also associated with increased arterial stiffness as shown by increases in peak wave velocity. Menopause accentuates the normal age-related increase in brachial-ankle pulse wave velocity (97), a measure of central and peripheral arterial stiffness that correlates with aortic arterial stiffness (98). This effect may relate to the anti-inflammatory properties of oestrogen as it appears to be induced by tumour necrosis factor-α (99).

Finally, oestrogen may mitigate endothelial dysfunction through its antioxidant properties, whereby it decreases oxidative stress and proinflammatory cytokines, but also increases the scavenging of reactive oxygen species (85,87,100). Reactive oxygen species can contribute to the emergence of hypertension by increasing vasoconstriction, promoting vascular smooth muscle cell proliferation and hypertrophy, and inducing inflammation (101).

1.2.1.2. Diabetes mellitus.

Oestrogen deficiency at menopause is associated with a reduction in metabolic rate, increased central adiposity, insulin resistance and dyslipidaemia, which work together to increase the

risk of diabetes (102-104). The loss of oestrogen's antioxidant actions is also likely to augment the contribution of inflammation and oxidative stress to insulin resistance. Postmenopausal oestrogen therapy improves glucose control in women with diabetes and improves insulin sensitivity (105,106).

Oestrogen appears to directly modulate glucose homeostasis by ERα-induced increases in insulin synthesis in the pancreas (104,107); increasing the glucose transporter protein GLUT4 expression and therefore glucose uptake in adipocytes (108); decreasing insulin resistance in skeletal muscle (102); and suppressing gluconeogenesis in the liver (109,110). ERβ appears to have an opposing effect with ERβ knockout mice displaying improved insulin sensitivity and glucose tolerance (106). Again, the relative abundance of the two receptor subtypes appears to define tissue responsiveness to oestrogen. For example, males have lower ERα:ERβ in their visceral fat depots and for females this ratio is reversed, which may explain the difference in spatial accumulation of fat between the two sexes and the change to visceral fat deposition post-menopause (102).

1.2.1.3. Dyslipidaemia.

At menopause, the favourable lipid and cholesterol profile observed in premenopausal women changes into one that is proatherogenic (111-117) and does not appear to be altered by ethnicity (113,115). Abrupt increases are observed in total cholesterol (111,114,116,117), low-density lipoprotein (LDL) (111,113,114) and triglycerides (111,114) during the menopause transition. High-density lipoprotein (HDL), which is anti-atherogenic, appears to remain relatively unchanged. The HDL subfraction HDL_2, appears to mediate the favourable effects of HDL whereas subfraction HDL_3 may be pro-atherogenic (112). Measurement of both these subtypes shows an initial decrease of HDL_3 at menopause with a gradual increase back to premenopausal levels (114).

Decreased oestrogen is associated with increases in hepatic lipase which hydrolyse triglycerides and LDL producing small dense LDL, and decreases HDL_2 in favour of increases in HDL_3 (112). Additionally, oestrogen decreases lipogenesis and fatty accumulation in the liver (110). Oestrogen may reduce circulating HDL indirectly. Oestrogen increases central adiposity which in turn negatively affects HDL concentrations. Therefore, decreases in HDL may be a secondary effect of increases in visceral fat (118,119).

1.2.1.4. Heart failure.

Heart failure is defined as "the inability of the heart to supply the peripheral tissues with the required amount of blood and oxygen to meet their metabolic demands" (120). Sex is associated with interesting differences in the epidemiology of this disease. Women usually present post-menopause, have preserved ejection fraction, have more functional impairment and have systolic hypertension and diabetes as important risk factors for the pathogenesis of heart failure (121). They also have more favourable outcomes. In spite of the later onset of disease in women, the lifetime risk for heart failure is still high at 1 in 5 for every decade from age 40 years (122).

The possible molecular mechanisms which underlie sex-related differences in heart failure may relate to genetic mechanisms, epigenetic mechanisms, and sex hormone-mediated differences (123). However, the increased risk of heart failure associated with increased systolic blood pressure (122,124,125), arterial stiffness (125), inflammation (126-129), insulin resistance (130), diabetes mellitus (124) and central obesity (131) suggest that oestrogen loss at menopause may contribute to its development.

1.2.1.5. Reduced renal function.

Oestrogen is reno-protective. A recent cohort study in 1,653 aged-matched women who underwent bilateral oophorectomy before the age of 50 years, showed that bilateral oophorectomy was associated with an increased risk for the development of chronic kidney disease by an estimated glomerular filtration rate-based definition (HR 1.42; 95% CI 1.14-1.77) (132). The risk was greater for women who underwent oophorectomy up to the age of 45 (HR 1.59; 95% CI 1.15-2.19). The mechanisms underpinning this are obscured, at least in part, by the often opposing actions of the oestrogen receptors ERα and ERβ (133,134).

Oestrogen mediates its protective effect on the kidneys indirectly through the modulation of the risk factors hypertension, diabetes mellitus, and inflammation; through its effect on the RAAS; and directly through oestrogen receptor interactions on mesangial and vascular smooth muscle cells (94). In the former, oestrogen increases mesangial production of the metalloproteinases MMP-2 and MMP-9, and decreases the production of Type I and IV collagen (135,136). In this way, oestrogen is thought to impede the progression of renal disease that is associated with matrix accumulation and fibrosis. Interestingly, oestrogen therapy is no longer effective once scarring has occurred, demonstrating the "window phenomenon" in postmenopausal oestrogen treatment.

1.2.1.6. Asymptomatic carotid disease.

Carotid artery atherosclerotic plaque size, measured as carotid intima-media thickness (IMT), is higher in women undergoing early menopause compared to those with late menopause (137). Oestrogen can modulate many of the risk factors that are positively correlated with IMT: serum lipids, body mass index, pulse pressure, systolic blood pressure, insulin resistance and serum glucose (138,139). However, the acute loss of oestrogen at menopause may have an additional as yet unidentified role in the increase in IMT as shown in an

ancillary study to the Study of Women's Health Across the Nation (SWAN) (140) . In these 249 women, the late peri-menopausal stage (defined as three consecutive months of amenorrhea) conferred a higher rate of change in IMT (mm/year) compared with the pre and early-perimenopausal stages, which persisted after adjustment for systolic blood pressure, body mass index, plasma LDL and age.

1.2.1.7. Severe mental illness.

The menopausal transition is associated with an increase in incident major depressive episodes and schizophrenia. In addition, it is associated with an increased risk for relapse in those with a past history of illness, and for symptom exacerbation, especially in women with schizophrenia (141-143). The influence of menopause on these findings not only relates to the decrease in oestrogen, but also to the loss of the cyclical hormonal input with which the female brain has become accustomed, requiring flexible adaptation through the hormonal fluctuations that define the perimenopausal period (143). Oestrogen is able to protect against the development of mood disorders through beneficial influences on serotonergic, noradrenergic and dopaminergic pathways (143-146); through the maintenance of grey matter (147); and through the reduction of oxidative stress (146).

1.3. Bone biology.

Bone has many functions in the body. It provides the structural form from which the attachment of muscles, tendons and ligaments allow movement; and it provides protection to organs such as the brain and spinal cord. Additionally, it is the home of the adult haematopietic system; and serves as a mineral reservoir containing about 99% of the body's calcium and 80% of the body's inorganic phosphate (148).

1.3.1. Basic multicellular unit.

The basic multicellular unit is responsible for bone turnover – a dynamic process of constant remodelling throughout the life of the vertebrate organism. This occurs with the synchronized action of osteoclasts and osteoblasts, cells that resorb and deposit bone, respectively. Osteocytes provide distant oversight to the resorption of bone by osteoclasts which is coupled to the subsequent bone formation by osteoblasts. After laying down new matrix, osteoblasts may undergo apoptosis, become embedded in matrix as osteocytes, or become lining cells of the bone surface. Upon activation, these lining cells prepare the bone surface by resorbing the underlying osteoid exposing the mineralized matrix for resorption by osteoclasts. Osteoclast progenitor cells, located in the bone marrow, are stimulated to differentiate and fuse together forming the functioning multinucleated cells. These then migrate to and bind with the bone surface through integrins, to establish controlled polarized resorption along their basal surface. Osteoblasts then produce osteoid, the unmineralized organic scaffold, which re-fills these resorption pits and subsequently undergoes mineralization to form inorganic hydroxyapatite (149).

A number of mechanisms match the resorptive activity of osteoclasts to bone formation by osteoblasts. Osteoclast resorption of the mineralized osteoid releases matrix-bound transforming growth factor-β and insulin-like growth factor-I stimulating osteoblast function.

Osteoclasts also secrete the Wnt ligand Wnt10b, bone morphogenetic protein-6 and sphingosine-1-phosphate which also stimulate osteoblast activity (150).

The interactions of RANK, RANKL and OPG are important in coupling osteoclastogenesis with bone formation. Receptor-activator of nuclear factor kappa beta (RANK) expressed on osteoclast progenitors, and its membrane-bound ligand (RANKL) expressed by osteoblast and osteoblast precursors, as well as secreted by T cells, interact to promote osteoclastogenesis. The osteoblast and osteocyte secreted decoy receptor, osteoprotegerin (OPG), binds RANKL with high affinity inhibiting its stimulatory effects on osteoclasts. In this way, cells of the osteoblast lineage influence osteoclast differentiation through the relative expression of RANKL and OPG (151).

Ephrin signalling between the osteoclast and the osteoblast is bidirectional and acts both as a coupling stimulator and coupling inhibitor. Ephrin B1 and ephrin B2 ligand expression on osteoclasts interacts with the receptor EphB4 on osteoblasts to forward stimulate osteoblast differentiation, and backward inhibit osteoclast differentiation. Additionally, acting as a coupling inhibitor, the osteoclast ligand ephrinA2 interacts with its receptor EphA2 on osteoblasts to inhibit osteoblast differentiation but stimulate osteoclast differentiation (152,153). Semaphorin signalling provides a further mechanism for uncoupling. Semaphorin 4D expressed by osteoclasts binds to its osteoblast-bound receptor Plexin-B1, suppressing bone formation (154).

Multiple influences of cellular (e.g. osteocyte and immune cells), hormonal (e.g. parathyroid hormone and parathyroid hormone-related protein), and mechanical origin, fine tune these actions leading to net bone formation or bone loss.

1.3.2. Signalling pathways.

Runt-related transcription factor 2 (RUNX2) is a key transcription factor for osteoblastic differentiation. Along with its direct gene target osterix, and nuclear factor for activated t cell 2 (NFATc2), it is responsible for the differentiation of osteoblasts and the expression of the marker proteins osteocalcin, collagen type I, osteonectin and osteopontin and alkaline phosphatase.

1.3.2.1. BMP and TGF-β.

Bone morphogenetic proteins (BMP) and transforming growth factor-β (TGF-β) are two of at least five ligand groups that make up the TGF-β superfamily. The receptor complex is comprised of two Type I receptors and two Type II receptors which are held together by the signalling ligand. Type II receptors then phosphorylate Type I receptors enabling interaction and subsequent phosphorylation of their intracellular signalling molecules, the Smads. Intracellular Smads are divided into the receptor activated R-Smads, Smad1, -2, -3, -5, -8; the CoSmad, Smad4; and the inhibitory I-Smads, Smad6 and -7. Once activated, the R-Smads complex with Smad4 and translocate to the nucleus where they alter chromatin remodelling and thereby facilitate the subsequent recruitment of transcription factors to target gene promoters. BMP signals through Smad1, -5, -8, whereas TGF-β signals through Smad2/3 (155,156).

Multiple factors contribute to the great diversity observed in the downstream effects of ligand binding. Chordin, noggin and Dan bind BMP and therefore prevent receptor binding. There are seven Type I receptors, and five Type II receptors, the combinations of which define intracellular effects. Additionally, some ligands require co-receptors such as betaglycan and endoglin for receptor signalling. Smad6 and Smad7 act by promoting receptor degradation, through Smurf1 and Smurf2, and by binding to Smad4 preventing it from forming complexes

with the R-Smads. Therefore, the relative concentrations of ligands and ligand antagonists, receptor combinations and co-receptor availability, as well as the intracellular Smad machinery define the intensity of convergence on stimulatory and inhibitory gene transcription (157). A further contribution to the signalling diversity is the activation of non-Smad signalling pathways such as the p38 MAPK pathway which activate the transcription factors c-Jun and c-Fos (158).

In bone, these signalling pathways converge on RUNX2. BMP stimulation increases alkaline phosphatase activity and mineralization and through upregulation of osteoblast RANKL, increases osteoclastogenesis (159). TGF-β promotes osteoblastic differentiation, matrix production and decreases RANKL production which shifts the balance of coordinated bone activity towards bone formation. Through Smad-independent pathways, it increases osteoprogenitor proliferation and supports the early differentiation of mesenchymal stem cells towards an osteoblastic lineage (156).

1.3.2.2. Wnt.

The Wnt/β-catenin signalling pathway plays an important role in bone homeostasis. Receptor activation by Wnt ligands triggers multiple signalling cascades essential for embryonic development and tissue regeneration. There are at least three pathways through which Wnt can signal, one such mechanism is the canonical pathway resulting in translocation of β-catenin to the nucleus. In the absence of Wnt binding, a destruction complex comprising Axin, APC, PP2A, GSK3 and CK1α enable GSK3 to phosphorylate β-catenin targeting it for degradation. Wnt ligands bind to the transmembrane molecules LRP5/6 and Frizzled allowing cross-linkage. This recruits the “destruction complex” via Axin, phosphorylating the intracellular tail of LRP5/6 leading to the intracellular binding and activation of Disheveled, which in turn prevents GSK3 action. β-catenin is therefore released,

accumulating in the cytoplasm and localizing to the nucleus where it acts as a transcription factor in conjunction with T cell factor/Lymphoid enhancer factor (Tcf/Lef) (160).

The activity of the Wnt/β-catenin pathway is modulated by a number of Wnt antagonists. Sclerostin formed predominantly by osteocytes, and Dickkopf factors (Dkk), bind to the Wnt binding site of Lrp5/6 inhibiting Wnt interaction. SFRPs (secreted frizzled related proteins) and Wnt inhibitory factor 1 (Wif1) bind to Wnt directly inhibiting interaction with membrane Frizzled (160).

In osteocytes, Wnt stimulation leads to downstream effects favouring bone formation. It promotes cell survival and an increase in connexin 43 which promotes cell to cell communication as well as the extrusion of protein messengers through hemichannels into the canaliculae (161-163). Wnt binding to osteocytes also results in increased OPG production (164) inhibiting osteoclast function as well as a decrease in sclerostin production which results in permissive Wnt signalling in osteoblasts promoting osteoblast differentiation and survival, and osteoclast inhibition (165,166). In osteoclasts Wnt signalling supports osteoclast progenitor proliferation with increased signalling important early on in the stimulation of precursors from a quiescent to proliferative phase, but is suppressed in the switch from proliferation to differentiation (167,168).

TGFβ, Wnt and BMP activity each promote osteoblast function however, their varied actions on RANKL expression in the osteoblast mean that TGFβ and Wnt signalling favour net bone formation, whereas BMP favours net bone loss.

1.3.2.3. Prostaglandin E_2.

Although there are four prostanoid receptors (EP), in osteocytes and osteoblasts prostaglandin E_2 (PGE_2) appears to signal predominantly through the G-protein couple receptors, EP2 and EP4 (169,170). The intracellular G-protein element is a trimer constituting α, β, and γ subunits. Upon activation of the receptor the α subunit and the βγ complex dissociate,

released to stimulate their downstream effectors. Both EP2 and EP4 receptors possess a stimulatory α unit ($G_s\alpha$) which increases intracellular cAMP levels through adenylyl cyclase (AC). cAMP acts through Protein Kinase A (PKA) with its downstream transcription factor CREB (171-173).

Unlike EP2, EP4 also possesses an inhibitory G-protein ($G_i\alpha$) leading to attenuated cAMP production at least initially, and phosphoinositide 3-kinase (PI3K) action leading to crosstalk with the Wnt pathway in stabilization of GSK3/β-catenin with subsequent β-catenin induced gene transcription (172,174).

In bone cells, PGE_2 stimulation can result in bone formation or bone resorption depending on the balance of cells involved. In osteoblasts, PGE_2 increases mineralization, differentiation in bone marrow-derived cells, and an altered RANKL:OPG expression favouring RANKL resulting in an upregulation of osteoclastic activity via cAMP and PKA pathways (175). Conversely in the osteocyte, EP2 and EP4 stimulation results in promotion of osteocyte survival, downregulation of sclerostin production, and a decrease in RANKL:OPG expression. This results in permissive osteoblastic Wnt signalling with an increase in the OPG to RANKL ratio inhibiting osteoclast action. It also increases connexin 43 and therefore the number of functional gap junctions (176).

1.3.2.4. Oestrogen.

Pre-menopause, the net effect of oestrogen signalling is maintenance of bone. It does this by decreasing the activity of osteoclasts, by promoting the activities of osteoblasts and osteocytes, and through dampening the effects of oxidative stress and inflammation which promote osteoclastogenesis and apoptosis of the osteoblast and osteocyte.

Through interaction with its receptors on osteoclasts and osteoclast progenitors, oestrogen increases apoptosis (177-179) and inhibits the downstream intracellular activities of RANKL

signalling preventing differentiation (180,181). It also mediates osteoclast survival and activity through altering the RANKL:OPG ratio expressed in osteoblasts and osteocytes favouring OPG (182-185), by decreasing reactive oxidative species and the inflammatory cytokines IL-7, IL-1, IL-6, TNFα (186,187) ; and by increasing TGF-β (188-190).

In osteoblasts, oestrogen promotes commitment to the osteoblast lineage, differentiation (191), survival (192), proliferation (193) and through its inhibition of NF-κB (187,194) promotes the elaboration of bone matrix (43). As well as stimulating osteocyte survival (192), it may also reduce the production of the Wnt antagonist, sclerostin (193).

Menopause heralds an increase in bone turnover with an increase in active resorption sites coupled with an increase in bone formation, however, the osteoblast is unable keep pace with osteoclast activity and bone loss ensues (43).

1.3.2.5. Parathyroid hormone.

There are two modal patterns of parathyroid hormone (PTH) secretion: a basal tone which comprises approximately 70% of secreted PTH, and superimposed low amplitude, high-frequency bursts accounting for the remaining 30% (195,196). There is also a circadian rhythm probably induced by diet and a circannual rhythm due to vitamin D production and increasing daylight hours in summer (195).

The effect of PTH on bone is both anabolic and catabolic. The frequent, low amplitude PTH bursts are thought to be responsible for its anabolic effect on bone. *In vivo* and *in vitro* studies of PTH administered intermittently promote osteoblast activation, differentiation, proliferation, inhibition of osteoblast and osteocyte apoptosis, and a reduction in sclerostin production from osteocytes (197,198). PTH can also increase osteoblast expression of the stimulatory ligand ephrin B2 (199,200). Together, these factors promote new bone formation (201).

The catabolic effects of PTH on bone, mediated by continuous PTH secretion, are through its ability to increase receptor activator of nuclear factor-kappa B ligand (RANKL) and decrease OPG production in osteoblasts and osteocytes which promotes osteoclast differentiation and the activity of mature osteoclasts thereby inducing bone resorption (198,202,203). A further mechanism is the ability of PTH to increase the expression of the monocyte chemoattractant protein-1 in osteoblasts which promotes osteoclastogenesis (204). Both modalities of PTH secretion stimulate monocyte chemoattractant protein-1 expression but it is the tonic PTH secretion which, because of sustained stimulation, shows higher bone resorption.

Recent advances in osteoimmunology, the study of the interaction of the immune system with bone, provide evidence for a substantial role for T lymphocytes in mediating both the anabolic and catabolic effects of PTH on bone. Continuous PTH increases osteoclast activity by increasing the secretion of TNF-α, RANKL, IFN-γ, IL-1, IL-6 and IL-17 from T cells. Furthermore, intermittent stimulation of T cells by PTH stimulates the release of anabolic Wnt ligand, Wnt 10b (197). Assimilating the results of studies in T cell-deficient mice, Pacifici proposes that the effect of PTH on T cells and the subsequent interaction with osteoblasts and osteocytes, operates in a serial circuit regulatory mode, whereby T cells promote PTH responsiveness in osteoblasts and osteocytes (197).

1.3.3. Osteocyte – more than just a quiet observer.

Comprising over 95% of all bone cells in the adult skeleton, our perception of the osteocyte has changed from one of passive observer to key player in the regulation of bone homeostasis. Due to their unique position within the bone matrix osteocytes act as sensors and transducers of mechanical stimuli responding to bone loading and unloading, influencing and orchestrating osteoblast and osteoclast activity and therefore bone formation and resorption. They are able to positively and negatively remodel their surrounding matrix in

response to hormonal cues in order to provide calcium and phosphate during lactation. Finally, with a realization of the vast connectivity with the vasculature it is unsurprising that osteocytes enable bone to act as an endocrine organ playing an important role in phosphate homeostasis (163,205).

Osteocytes are cells of osteoblast lineage. It is unknown what triggers the osteoblast to differentiate into an osteocyte as opposed to undergoing apoptosis or becoming a surface lining cell, but with time the cell becomes embedded within the mineralized bone. During this burying process they can be identified from osteoblasts by the gradual expression of marker genes such as sclerostin, fibroblast growth factor 23 (FGF23) and oxygen-regulated protein (ORP150) that signal their change in morphology and function (206-208). Embedded within the osteoid-bound lacunae the osteocyte actively extends and retracts dendrites resorbing bone in order to do so. They do this via a membrane-bound matrix metalloproteinase, MT1-MMP (membrane-type matrix metalloproteinase 1), that cleaves collagen I, II, III, fibrin, fibronectin and other matrix molecules allowing dendritic expansion (209). These dendrites extend to the bone marrow where they are able to influence both osteoclast and osteoblast precursors, to the periosteum and endosteum where they influence the differentiation of osteoblasts, to other osteocytes promoting integrated function and to the vasculature enabling endocrine signalling.

Bathed by canalicular fluid, it is their ability to detect mechanical stress as well as an extensive network of dendrites that allows the osteocyte to be extremely effective in its role as mechanosensor and transducer (208).

The osteocyte is able to detect mechanical loading in three ways:

1. through direct physical deformity, more so of the dendrites than the cell body;

2. through the electric streaming of potentials with the flow of ionic canalicular fluid across charged cell surfaces; and

3. through the shear stress created by increased fluid flow within the canaliculi.

Shear stress may be sensed through cell surface primary cilia (210) of the osteocyte and of other differentiated and mesenchymal stem cells of bone, as removal of primary cilia from bone cells in vivo results in reduced loaded induced bone formation; the osteocyte cell body; or via dendrites with their surface glycocalyx.

In response to mechanical loading osteocytes release nitric oxide, ATP, Ca^{2+} and prostaglandin E_2 (PGE_2) which on balance favour bone formation. The simultaneous downregulation of the Wnt antagonists sclerostin, DKK1 (Dickkopf-related protein 1) and sFP1 provide a permissive environment for positive Wnt-related osteoblastic signalling (211). Formation of the RANKL decoy receptor OPG by the osteocyte prevents osteoclast activity further promoting bone formation. However, RANKL produced by the activated osteoblast allows targeted osteoclast activity in the area undergoing remodelling. BMP7 is also released in response to fluid shear stress (212). With inactivity and bone unloading, the balance shifts in favour of the Wnt antagonists. This together with formation of RANKL and M-CSF culminate in osteoclast differentiation and activity resulting in bone resorption. Mesenchymal stems cells and osteoblasts also sense and respond to mechanical stimuli. However, with their vast interconnectedness, the osteocyte is responsible for the coordinated response, enabling bone to act as a syncytium.

As promoters of osteoclast differentiation and function, osteocytes express RANK-L both in the membrane-bound and soluble form although the bound form appears necessary for osteoclastic support. They express a higher amount of RANKL than osteoblasts and therefore have a much greater capacity to support osteoclastogenesis (213).

In the osteocyte, some of the signals culminating in β-catenin translocation to the nucleus originate via Wnt signalling but others do not. These include oestrogen receptor activation by oestrogen and selective oestrogen receptor modulators (bisphosphonates and calcitonin) which chaperone β-catenin translocation to the cell nucleus, and PGE2 which acts via the EP2/4 receptor to free β-catenin from degradation by inactivating GSK-3 (214). The downstream effects of this are increased cell viability, increased connexon 43 (Cx43) formation resulting in increased cell to cell communication (214), and an increase in the OPG:RANKL ratio culminating in decreased RANKL signalling. The latter is evident in mice with osteocyte knockout of the β-catenin signalling pathway who display increased bone porosity secondary to reduced OPG expression and therefore increased RANKL signalling (164). Therefore, Wnt signalling although perhaps more correctly, β-catenin signalling, plays an important role in the far-reaching functions of the osteocyte.

1.3.4. Osteoblast.

Osteoblasts are derived from bone marrow stromal cells which have site-specific embryological origins. Although all bone marrow stromal cells are derived from the mesoderm, the bones forming the vertebrae, ribs and part of the shoulder girdle arise from the somite layer of the lateral plate mesoderm; whereas the limbs, pelvis, sternum and remaining portion of the shoulder arise from the somatic layer of the lateral plate mesoderm (215). Osteoblasts are responsible for bone formation and perform this task in two ways, endochondral bone formation occurs by the replacement of a cartilaginous intermediary template, culminating in long bone formation; and intramembranous bone formation occurs directly from sheets of mesenchymal connective tissue, responsible for the formation of the flat bones of the cranium.

Osteoblasts elaborate the organic osteoid, 90% of which is Type I collagen, which forms the scaffold for subsequent mineralization. Osteoblasts also synthesize a number of non-collagenous proteins that are important for cell adhesion (osteopontin, bone sialoprotein), promote appropriate mineralization (bone sialoprotein, alkaline phosphatase), but prevent excessive mineralization (matrix Gla protein, osteocalcin). Through the expression of the RANKL and OPG, osteoblasts promote osteoclast differentiation and activity.

1.3.5. Osteoclast.

Osteoclasts originate from haematopoietic stem cells of the bone marrow monocyte/macrophage lineage. Embryologically, they originate from the splanchnic lateral plate mesoderm where they temporarily reside in the wall of the descending aorta before populating the haematopoietic organs (216). RANKL and macrophage colony-stimulating factors (M-CSF), synthesized by both osteoblast and osteocytes as well as their precursors, are crucial ligands for stimulating differentiation, the fusion of precursor cells to form the multinucleated mature osteoclast, and to generate the necessary intracellular machinery to enable bone resorption. T cells also secrete soluble RANKL.

Nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) is the master transcriptional regulator for the transcription of genes necessary for osteoclast activity. Osteoclasts have a gene complement that is both NFATc1 regulated and NFATc1 augmented (217). Through intracellular actions of TNF receptor associated factor (TRAF)-6 and induction of sustained low amplitude calcium oscillations that activate calcineurin, RANKL binding stimulates NFATc1 activity. NFATc2 is also present in osteoclasts, but the selective auto-amplification of NFATc1 and the muting of the *NFATc2* promoter by epigenetic modifications, means that NFATc1 plays the role of master transcriptional regulator (218). IL-1 also stimulates osteoclastogenesis but in a permissive RANKL environment. In inflammatory osteolysis,

TNF-α also increases NFATc1 through both M-CSF and RANKL binding, but this is via TRAF2 (217). Epigenetic modifications are also required for NFATc1 activity. DNA methylation, NFATc1 acetylation by histone acetyl transferase, and short non-coding RNAs all play a role in osteoclastogenesis. In addition to the promoters of NFATc1 action, there are many negative regulators which act by downregulating the expression of NFATc1 (219).

Once stimulated, osteoclasts must undertake a number of processes necessary for effective resorption. Matrix to cell signalling occurs between the integrin αvβ3 to amino acid sequences present in matrix proteins such as osteopontin and bone sialoprotein, initiating a conformational change which stimulates organization of the cytoskeleton and the formation of an actin ring (220). This actin ring, or sealing zone, which creates a sealed environment within which osteoclasts can function, is crucial for effective resorption. It is comprised of actin podosomal units which through interconnectivity, form a podosomal belt (221). The formation of the ruffled border signifies the insertion of membrane machinery, H^+-ATPase and Cl^- channel, which permits acidification of the resorptive zone between the polarized osteoclast and the bone surface. H^+ is generated through the activity of the enzyme carbonic anhydrase II on CO_2 (222). This acidification liberates the mineral from bone leaving behind the collagen scaffold which is degraded through lysosomal release of cathepsin K (223). Along with facilitating osteoclast migration, matrix metalloproteinases also promote the degradation of the organic matrix (224). The osteoclast processes the resorbed bone elements before releasing them into the surrounding extracellular fluid (225,226).

1.4. Calcium.

Calcium regulates a large number of diverse physiological processes. It is important in nerve conduction, skeletal and cardiac muscle contraction and coagulation, and is a vital secondary messenger in cell communication. Symptomatically, hypocalcaemia can result in neuromuscular irritability with tetany, muscle twitching, seizures, and life-threatening laryngo- and bronchospasm (227). Hypercalcemia similarly affects neuromuscular excitement and nerve conduction pathways with an increased risk for abnormal cardiac rhythms that may be fatal, and in a normal pH environment, may increase coagulability (228,229). Within the cell, uncontrolled increases in calcium can initiate apoptosis (230). Therefore, to manage its intracellular effects, the intracellular concentration of calcium is governed by the interaction of plasma membrane calcium channels, intracellular calcium channels like those in the endoplasmic reticulum, and calcium-binding proteins. It is not surprising that the control of plasma calcium is also tightly regulated.

1.4.1. Calcium homeostasis.

Maintenance of plasma calcium is achieved by the coordinated interaction between intestinal calcium absorption, excretion or reabsorption in the kidneys, and the balance between bone resorption or formation which can release or remove calcium from the circulation. Hormonal oversight is provided by the calciotropic hormones parathyroid hormone (PTH), calcitonin and also vitamin D with indirect contributions of fibroblast growth factor-23 and its co-receptor Klotho.

1.4.1.1. Parathyroid hormone.

PTH is responsible for increasing serum ionized calcium, which it does by binding to PTH1R receptor in bone and kidney. Calcium sensing receptors on the surface of parathyroid cells sense the circulating concentration of ionized calcium. When ionized calcium is elevated,

calcium binding initiates an intracellular calcium burst inhibiting PTH secretion (201). Conversely, low plasma calcium is permissive for PTH secretion.

There are two modal patterns of PTH secretion: a basal tone which comprises approximately 70% of secreted PTH, and superimposed low amplitude, high-frequency bursts accounting for the remaining 30% (195,196). There is also a circadian rhythm, probably induced by diet, and a circannual rhythm due to vitamin D production and increasing daylight hours in summer (195).

1.4.1.2. Vitamin D_3.

Vitamin D_3 can be absorbed from the diet but is more commonly produced in the skin by ultraviolet irradiation of 7-dehydrocholesterol. It is then transported to the liver, bound to vitamin D binding protein, where it begins its transformation into an active hormone. Here it undergoes hydroxylation producing 25-hydroxyvitamin D_3, dependent on cytochrome P_{450}. All four isoforms of this enzyme are able to perform the hydroxylation but the CYP2R1 isoform may be the most important (231). In the kidney, 25-hydroxyvitamin D_3 is further hydroxylated to its active form of 1,25-dihydroxyvitamin D_3 (calcitriol) or to the inactive 24,25-dihydroxyvitamin D_3. Calcitriol then interacts with the vitamin D receptor in target cells in the intestine, kidney and bone initiating responses to increase plasma calcium (232,233). The rate-limiting step in formation of calcitriol appears to be at the kidney with CYP27B1, also called 25-hydroxyvitamin D-1α-hydroxylase. Calcitriol provides its own negative feedback by suppressing the action of the 1α-hydroxylase and stimulating the action of 24-hydroxylase producing 24,25-dihydroxyvitamin D_3 (234).

1.4.1.3. Calcitonin.

Calcitonin is encoded by the *calca* gene which, by tissue-specific alternative RNA splicing, also encodes calcitonin gene-related peptide (CGRPα) and katacalcin (235). It is produced by

parafollicular or C cells of the thyroid gland in response to elevations in plasma calcium (236). Calcitonin is also secreted by other tissues but these do not appear to be affected by changes in plasma calcium (237,238). Different calcitonin receptor (CTR) isoforms and binding of various receptor-activity-modifying-proteins (RAMPS) provide tissue specific phenotype of ligand interaction and dictate ligand specificities. Further intracellular variability is provided by the different GTP-regulated G protein family machinery with which the CTR interacts (238-240). Besides its effect in calcium homeostasis, calcitonin is implicated in many other activities including analgesia, decreasing appetite and decreasing gastric acid secretion (238).

1.4.1.4. Intestinal absorption.

Intestinal absorption of calcium occurs through two mechanisms: the paracellular pathway and the transcellular pathway. Calcium absorption through the paracellular pathway is dependent on a concentration gradient between gut lumen and plasma and is thought to occur through the length of the small intestine when dietary calcium is adequate or increased (241). The transcellular pathway appears to be saturable and is responsible for calcium absorption in the duodenum and jejunum in the setting of a low calcium diet (242). The duodenum has the greatest capacity for absorption but most calcium is absorbed in the ileum because of the longer transit time here of approximately two hours versus two minutes in the duodenum of rats. (242).

Absorption through the paracellular pathway occurs through tight junctions that guard the gaps between cells. Positioned predominantly at the apical surface of enterocytes they are made up of various proteins, including claudins. Claudins 2, 12 and 15 are responsible for forming ion pores that allow the transport of calcium through this pathway (243,244). These pores allow for passive diffusion of calcium from the gut lumen governed by the

electrochemical gradient between lumen and plasma. This gradient is created by dietary calcium intake, by a secondary concentration gradient driven by Na^+ absorption and the consequent osmotically driven water absorption, and finally by solvent drag, also dependent on water absorption (245). Therefore, those factors that affect Na^+ transport will also have a secondary effect on calcium absorption.

Transcellular absorption of calcium occurs in the apical surface of gut epithelial cells through TRPV6 (transient receptor potential, vanilloid type). Once in the cell cytosol, calcium is bound to calbindinD_{9k} which facilitates movement to the basolateral membrane and, in its binding, buffers intracellular free calcium concentration. Transport out of the cell occurs through the plasma membrane calcium-ATPase ($PMCA_{1b}$) and the sodium-calcium exchange pump (NCX1) (246). The apical L-type channel $Ca_v1.3$ also facilitates calcium absorption (247-249). The two calcium absorption receptors, TRPV6 and $Ca_v1.3$, are thought to act in complementary roles with $Ca_v1.3$ active in the depolarized situation of digestion and TRPV6 active in the hyperpolarized environment between meals. Interestingly, $Ca_v1.3$ is expressed throughout the intestinal tract challenging many of the paradigms regarding location of calcium absorption (247).

When considering dietary calcium absorption from the intestine it is important to remember that the intestine also has a secretory function and calcium secretion is part of this (250). Different segments of bowel display net absorption or net secretion depending on the calcium that is available in the diet. Therefore, assessments of dietary calcium absorption efficiency are based on the calcium that is administered in the diet and hence are over and above that which is reabsorbed for normal intestinal secretion. That being said, 20-60% of dietary calcium is absorbed (251). Dietary proteins, especially those present in milk, improve the availability of calcium for absorption whereas some substances bind calcium (oxalate, phytic acid and phosphate) and decrease its availability (252,253). Absorption efficiency is

increased in situations of low dietary calcium and is reduced when dietary calcium is high (251), and modulated depending on physiological requirements. In infancy, calcium absorption is about 60% from breast milk, which may be promoted by lactose and other milk proteins. In children, low dietary calcium leads to increased absorption efficiency (60%) even in the presence of phytic acid (254). The demands of pregnancy also improve absorption efficiency (255,256). Consequently, calcium absorption is not a linear function of dietary intake since the body possesses the regulatory pathways to increase absorption when required.

Vitamin D modulates all aspects of TRPV6- mediated transcellular absorption and also has favourable effects on the paracellular pathway (244,257). Calcitriol (1,25 dihydroxycholecalciferol), the active metabolite of vitamin D3, increases the receptor and transporters TRPV6, $PMCA_{1b}$ and NCX1, as well as the calcium binding protein calbindinD_{9k}, and thereby increases calcium absorption. It also increases Claudin-2 and Claudin-12 which facilitate absorption of calcium through the paracellular pathway (244).

Decreased pH, provided by the action of intestinal alkaline phosphatase at the brush border, is thought to provide a minute by minute control of calcium absorption. Increased luminal concentration of calcium promotes alkaline phosphatase action inducing a decrease in local pH (258). This relatively acid environment reduces calcium absorption (258) which may represent a decrease in calcium-receptor binding as low pH is known to decrease calcium binding. PTH also influences calcium absorption in the intestine through its effect on pH and induction of electrogenic ion transport (259,260). *In vivo* studies of intestinal cells show that PTH stimulation increases apical bicarbonate secretion, and therefore pH, and enhances electrogenic ion transport which together would promote calcium absorption.

Oestrogen is able to increase the action of TRPV6 in the intestine, promoting calcium absorption (261,262).

1.4.1.5. Renal calcium handling.

Approximately 98-99% of filtered calcium is reabsorbed in the kidneys. 60-70% of this occurs in the proximal convoluted tubule where 80% of calcium absorption is paracellular and driven by solvent drag. The thick ascending loop of Henle accounts for ~20% of calcium reabsorption and occurs through both paracellular and transcellular pathways. In the distal convoluted tubule and collecting duct, accounting for ~15% of total renal reabsorption, calcium is reabsorbed exclusively through the transcellular pathway.

Similar to calcium absorption from the intestine, renal reabsorption of calcium through the transcellular pathway requires receptor mediated transport across the apical membrane through TRPV5 (transient receptor potential, vanilloid type), cytosolic chaperoning via a calcium binding protein calbindin-D_{28k}, and finally extrusion through the basolateral membrane via sodium-calcium exchanger NCX1 and the plasma membrane calcium-ATPase $PMCA_{1b}$. Intracellular buffering provided by calbindin-D_{28k} is important as increased cytosolic calcium inhibits TRPV5 activity (263). Paracellular transport is facilitated by the presence of claudin-16 and claudin-19, however, stimulation of the calcium sensing receptor increases expression of claudin-14 impeding permeability (234,264,265).

Both PTH and calcitriol are able to enhance the renal absorption of calcium. PTH can induce a fast increase in transcellular calcium reabsorption by PKA-dependent phosphorylation of the TRPV5 channel which enhances its open probability (266). Like calcitriol, it can increase the expression of the calcium transport proteins TRPV5, calbindin-D_{28k}, and NCX_1 (267-269) therefore providing both short-term and longer-term increases in calcium absorption. Unlike calcitriol, PTH is also able to increase the expression of $PMCA_{1b}$ in the basolateral

membrane, and stimulate a translocation of claudin-14 from the membrane to the cytosol thereby preventing its negative effects on calcium reabsorption (268,270).

Further mechanisms controlling calcium absorption in the kidney include intracellular calcium concentration which has a negative feedback on TRPV5 activity; extracellular pH, with alkalosis increasing TRPV5 activity and acidity decreasing it; and Klotho, which increases TRPV5 retention in the cell membrane and therefore increases calcium reabsorption (263).

FGF-23 has indirect effects on calcium homeostasis many of which occur in the kidney. Osteoblast and osteocyte derived fibroblast growth factor-23 is stimulated by calcitriol and increased plasma concentrations of calcium. In the kidney it promotes phosphate excretion, downregulates its co-receptor Klotho, and promotes 24-hydroxylation of 25-hydroxycholecalciferol over the action of 25-hydroxyvitamin D-1α-hydroxylase, thereby reducing calcitriol (271,272).

Oestrogen can increase the expression of all calcium transporter proteins involved in transepithelial transport in the kidney (263).

1.4.1.6. Role of bone in calcium homeostasis – the impact of calcitropic hormones.

Approximately 99% of body calcium is stored in bones and teeth. Here, with phosphate, it is predominantly in the form of hydroxyapatite with adsorbed magnesium (273). When bone is resorbed to provide calcium to buffer plasma concentrations, provision must be made for the excess phosphate and magnesium that is released as well as a dampening mechanism to prevent excessive bone loss. This action of bone to buffer plasma calcium must also fit into the bigger picture of bone accrual during growth and maintenance throughout life to enable

effective bipedal activity. PTH, calcitonin, calcitriol and FGF-23 provide this oversight which can be fine-tuned by the action of additional hormones. An important feature of bone activity is the coupling of osteoblast and osteoclast function, with osteocytes modulating activity in the context of pressure inputs. Therefore, modulation of one bone cell type will have downstream effects on the other.

Please see bone biology section for a discussion of PTH action on bone.

Vitamin D acts on bone to increase serum calcium. It does this by increasing osteoclast number and function and by increasing RANKL expression on osteoblasts thereby increasing bone resorption. It also induces FGF-23 production in osteocytes which downregulates further production in the kidneys.

Calcitonin is thought of as "guardian of the skeleton" (274) and appears to have its greatest protective effect on bone at times of high calcium demand such as in growth, pregnancy, and lactation (275) where it tempers PTH-induced bone resorption. It is thought to have little effect on the aging skeleton.

In vitro studies of osteoclast cultures show that calcitonin inhibits osteoclastogenesis (276), and in mature osteoclasts induces loss of the ruffled border and decreased cell motility ultimately resulting in reduced osteoclastic bone resorption (240,277,278). Two logical assumptions can be made from this finding. The first is that reduced levels of circulating calcitonin might result in bone loss. This is not the case in individual's post-thyroidectomy who did not show a decrease in bone mineral density. This could be explained by a possible upregulation of calcitonin secretion from extrathyroidal sites as these patients had near normal basal plasma levels of calcitonin (279). Secondly, that increased calcitonin might result in an increased bone density, however, individuals with medullary thyroid cancer with elevated levels of circulating calcitonin have normal radial bone densities and reduced spinal

densities (279). This could be explained by the negative feedback that calcitonin has on its own receptor (238). An observation that is difficult to explain based on the findings from osteoclast cultures is that mice lacking calcitonin and CGRPα have ***increased*** not decreased bone mass as may be expected (280). Here, part of the answer lies in the coupling of osteoclast and osteoblast activity: calcitonin prevents osteoclast-stimulated bone formation (281). Osteoclasts secrete sphingosine 1-phosphate through a secretory transmembrane protein which stimulates osteoblast function as well as an increased RANKL:OPG ratio. Calcitonin downregulates osteoclastogenic expression of this secretory transmembrane protein with accumulation of sphingosine 1-phosphate in the cytosol, thereby resulting in an initial uncoupling of osteoclast activity from bone formation, also preventing further osteoclastic stimulation by RANKL. An additional explanation surrounds the finding that osteoclasts possess a ryanodine receptor-like molecule in their plasma membrane which acts as a calcium sensor stimulated by higher ambient concentrations of calcium than the conventional calcium sensing receptor present in the parathyroid gland (282). Stimulation of the calcium sensor by calcium or other similar divalent cations (Mg^{2+}, Ni^{2+}) causes a release of intracellular calcium stores thereby increasing cytosolic calcium. This leads to osteoclastic cell retraction, inhibition of enzyme release and of bone resorption (283).

1.5. Calcium and the prevention of osteoporotic fractures.

Based on the assumption that increasing calcium intake was safe, effective, and necessary to maintain bone mineral density, calcium supplements have routinely been prescribed for the prevention of osteoporotic fractures where dietary calcium intake has fallen below national recommendations. Historically, these recommendations have been based on the outcomes of balance studies and the premise that all retained calcium is transferred to bone; as well as studies of the effect of calcium supplementation on bone mineral density and fracture risk, which are not all applicable to community dwelling postmenopausal women (48,284). Balance studies have limitations which render them unreliable as a measure of the effect of calcium on bone mineral content (285). Reid *et al.* re-analysed data from the Auckland Calcium Study to assess the relationship between the change in total bone mineral content over 5 years and dietary calcium intake (286). For comparison, the theoretical gains in bone mass from the calcium balance data of Heaney *et al.* is superimposed on this graphical relationship (287). The assumed gains in bone mineral content from this balance study do not correlate with observed real changes measured by dual-energy X-ray absorptiometry as illustrated in this comparison.

The assumption that calcium supplements are safe is also questionable. Evidence indicates that calcium supplements are associated with an increased risk for cardiovascular events (288,289), and side effects which contribute to the low treatment adherence in randomised controlled trials (290,291). Therefore, current dietary and supplemental recommendations for calcium intake must be justified through more tangible outcomes such as bone mineral density and fracture incidence.

1.5.1. Effect of calcium on bone mineral density.

The rate at which bone is lost in postmenopausal women depends on the site analysed and number of years since menopause (292). In women greater than five years post-menopause, this is about 1.4% per year at the total body, 1% at the lumbar spine, and about 0.4% at the femoral neck (293).

Recent studies do not support an association between dietary calcium intake and bone mineral density. In a study of 838 Italian women (mean age 62 years, standard deviation 14 years), and 162 Italian men (mean age 53.7 years, standard deviation 13.1 years), dietary calcium intake was not correlated with bone mineral density (294). In a further study of 323 men, dietary calcium intake by tertile (<660, 660-920, >920 mg/day) did not result in differences in bone mineral density at any site. In a smaller subset of this study, a longitudinal analysis was undertaken in 99 men who comprised the placebo group of the original trial. Here, the authors inquired as to whether bone mineral density loss over two years was related to calcium intake. They found no correlation between dietary calcium intake and bone loss at the spine, hip or total body. Additionally, a re-analysis of the Auckland Calcium Study data (295) also failed to detect a relationship between quintiles of dietary calcium intake and bone mineral content (286).

These smaller studies may be insufficiently powered to detect a true interaction between dietary calcium and bone mass. Kim *et al.* have recently published results analysing data from the Korean National Health and Nutrition Examination Survey, reporting beneficial effects of dietary calcium in the second quartile of intake (296). In 4,494 postmenopausal women, a dietary calcium intake in the range of >400 to ≤800 mg per day compared with an intake of ≤400 mg/day, was associated with statistically significant increases in the femoral neck in the fully adjusted model, and for lumbar spine in the partially adjusted model.

Additionally, in a meta-analysis of 15 randomised controlled studies of which 87% were women (297), increasing calcium intake from dietary sources also conferred beneficial effects on bone mineral density. Although it did not alter bone mineral density at the forearm, dietary calcium was associated with small increases at the remaining sites. Between-group differences in percentage change from baseline reached statistical significance at one and two years for both total hip (one year: 0.6%, two years: 1.5%) and total body (one year: 1.0%, two years: 0.9%) and for lumbar spine and femoral neck at year two only (0.7%, 1.8% respectively).

Randomised controlled trials of the effect of calcium supplements on bone mineral density, have consistently shown improvements with immediate effect in the lumbar spine and femoral neck amassing to differences in percentage change at one year of 1.7% at the lumbar spine and 1.1% at the femoral neck (293). The relatively high gain at these two sites compared with total body (between group difference of 0.4%) is thought to be secondary to the higher trabecular content of the bones at these sites, and the initial change in remodelling rate induced by calcium (298). Other studies have demonstrated a greater effect in women with dietary calcium intakes below 400 mg/day (292), and where vitamin D is co-administered in institutionalized patients with low serum vitamin D (48). However, gains in bone mineral density are not sustained once calcium supplementation ceases (299).

A meta-analysis also confirms the beneficial effect of calcium supplements on bone mass. Information regarding site specific bone mineral density from 51 randomised controlled trials of calcium supplements, with participant age >50 years at baseline and 94% female participation; was analysed at three times points: 1 year, 2 years, and >2.5 years. For the lumbar spine, the greatest differences were observed in the first year (1.2% and 1.4% respectively) which eroded over subsequent years. For the femoral neck, forearm and total

body, the largest difference in bone mineral density were observed at the final time point (1.5%, 1.8% and 0.8% respectively).

Overall, dietary calcium and calcium supplements are both beneficial for bone mineral density in healthy, postmenopausal women. Calcium supplements are more effective within the first year, showing greater percentage differences (an advantage of 0.6-1% over dietary sources) at all but the total body site. In the aforementioned meta-analysis, the lack of significance reached at some time points for the effect of dietary sources of calcium, may represent a lack of power as reflected in the large 95% confidence intervals. Furthermore, the forearm appears to be relatively resistant to gains in bone mass with dietary calcium alone, as bone mineral density measurements at one year showed no between group percentage difference, and at two years, an 0.1% difference. As the 95% confidence interval for the effect of calcium supplements on forearm bone mineral density exceeds that of the point value for the diet group, dietary sources of calcium are unlikely to reach the magnitude of the effect conferred by calcium supplements at this site.

1.5.2. Effect of calcium on fracture incidence.

Beneficial effects of calcium on bone mineral density are of no consequence if they do not lower fracture incidence. In their meta-analysis, Tang *et al.* suggest that the gains in bone mineral density do translate to a lower risk of fracture (300). In this study, the authors considered the effect of calcium with or without vitamin D on hip and vertebral bone mineral density as well as total fracture risk. In the 24 studies contributing bone mineral density information, calcium with or without vitamin D improved bone mineral density in the hip (difference in means 0.54; 95% confidence interval 0.35-0.73); and vertebral body (difference in means 1.19; 95% confidence interval 0.76-1.61). In their fracture analyses, calcium alone conferred a relative risk of 0.90 (95% confidence interval 0.80-1.00) and calcium in

combination with vitamin D, a relative risk of 0.87 (95% confidence interval 0.77-0.97). Subgroup analyses provided some useful insights. In analyses of studies that reported both bone mineral density and fracture data, they found that a ≥1% bone mineral density change was protective. They also report ≥80% compliance with study treatment protocols, baseline dietary calcium intakes <700 mg/day, and being institutionalized as subgroups where calcium with or without vitamin D provided increased protection against fracture incidence. Although serum 25(OH) vitamin D_3 was low in some participants, a subgroup analysis found this interaction to be insignificant.

Because this meta-analysis included studies comprised of participants who were institutionalized, the generalizability at a population level for the prevention of fracture is unknown. Additionally, the inclusion of institutionalized individuals with low serum 25(OH) vitamin D_3 (48) requires further mention. A recent re-calculation of these serum 25(OH) vitamin D_3 concentrations suggests that the true value was much lower than initially thought, raising concerns that these individuals had osteomalacia (301). Together, these suggest that calcium and vitamin D supplementation is beneficial for fracture prevention in institutionalized individuals with a low serum 25(OH) vitamin D_3 concentration, but does not establish the protective effect of calcium supplementation alone, or in combination with vitamin D, in the general, healthy, postmenopausal population.

In 2015, Bolland *et al.* published their results of the effects of dietary calcium and calcium supplements on fracture incidence (301). Only two randomised controlled trials assessing the effects of dietary calcium on fracture outcome were identified, with milk powder as the source in one study (n = 200) and a hydroxyapatite preparation in the other. In the former, number of fractures were low (1 in the calcium group versus 3 in the placebo group) with a resulting relative risk of 0.33 (95% confidence interval 0.04 to 3.2). In the latter study, information regarding fracture outcome was not reported separately from those of calcium

supplements. Given the inadequate number of identified randomised controlled trials, the authors analysed observational studies, comprised of 42 cohort studies. Amongst these studies, 58 associations between dietary calcium and fracture outcome were noted of which 74% showed a neutral relationship. Of the 12 inverse interactions for which there were relative risk estimates, 9 were considered weak associations as they had relative risks between 0.5 and 2.0. An important finding was that dietary calcium intakes in the recommended range of 1200 mg/day did not show a reduced risk of fracture, challenging the current dietary calcium intake recommendations for older people (302,303).

In the same study, Bolland *et al.* also assessed the effect of calcium supplements on fracture risk. 26 randomised controlled trials were pooled for analysis showing an 11% reduction in risk with calcium supplements for all fractures and a 14% reduction in vertebral fractures. The authors questioned the authenticity of these findings given what appeared to be an oversupply of small studies showing beneficial results. They therefore, performed subgroup analyses according to the risk of bias. In the groups with a low risk of bias, calcium supplements did not improve fracture outcome. The authors also questioned the generalizability of their findings given the results of the Chapuy study (48) which was of frail, elderly, institutionalised French women with low baseline dietary calcium intake and low baseline serum vitamin D concentrations, influenced the overall outcome. Bolland *et al.* therefore performed sensitivity analyses excluding this study and did not find a fracture benefit in those taking calcium supplements.

In 2017, Zhao *et al.* published a comprehensive meta-analysis of the effects of calcium intake and fracture incidence in community-dwelling adults older than 50 years old, the majority of who were women (49,304); and in doing so, brought resolution to the concern of generalizability. They investigated whether calcium supplementation, vitamin D supplementation, or the combination of calcium and vitamin D supplementation affected

fracture incidence. In the analysis of 33 randomised controlled trials, calcium supplementation alone had no effect on hip fractures (relative risk RR 1.53; 95% confidence interval CI 0.97-2.42; *n* = 10,307), non-vertebral fractures (RR 0.95; 95% CI 0.82-1.11; *n* = 10,193), vertebral fractures (RR 0.83; 95% CI 0.66-1.05; *n* = 3,835); or total fracture incidence (RR 0.88; 95% CI 0.75-1.03; *n* = 6,483). In the 13 trials comparing calcium and vitamin D supplementation with placebo or no treatment, no statistically significant effects were observed in fracture incidence. For both these analyses, subgroup analyses by calcium dose, sex, fracture history, dietary calcium intake, or baseline serum vitamin D did not affect the findings of the primary analyses.

A possible concern with this meta-analysis is the inclusion of the many studies with a follow-up duration of one year or less. For analyses of calcium supplementation use alone this is of no consequence as it only influences the analysis of total fractures, where the study of concern carries a weight of 0.5%. Similarly, although there are more of these studies included into the analyses for the effect of calcium combined with vitamin D supplementation, again the effect is likely to be small given the weight of the effect is 2% or less.

A further observation deserves mention. In this study, combined calcium and vitamin D supplementation had no effect on hip fracture incidence (risk ratio 1.09; 95% confidence interval 0.85-1.39). For calcium supplementation alone, the effect on hip fracture incidence was not statistically significant but given the populations involved were less than half of those in the combined calcium and vitamin D analysis, and the 95% confidence interval is wide and approaching significance (0.97-2.42), the authors acknowledge that the 50% increase in risk ratio could be a real phenomenon dampened by a lack of statistical power. Indeed, from at least 2008, there has been an awareness that calcium supplementation alone may in fact *cause* hip fractures (305).

1.5.3. Conclusion.

Increasing calcium intake, through diet or calcium supplementation, is associated with small increases in bone mineral density. However, this does not reduce the outcome of interest in postmenopausal women: fracture incidence. It is interesting that although calcium supplementation confers small improvements in bone mineral density at the lumbar spine and femoral neck within the first year, these do not translate to a lower fracture incidence at these sites and may actually increase hip fracture risk. Therefore, in the absence of evidence of fracture benefit, current dietary and supplemental recommendations for calcium intake require re-evaluation.

1.6. Calcium and the risk of cardiovascular disease.

Cardiovascular disease (CVD) poses a significant health problem in New Zealand. In 2015, ischaemic heart disease and stroke deaths combined, accounted for 23.6% of all deaths, second to cancer deaths comprising 30.2% (306). During 2000-2004 Maori death rates from CVD were 2.3 times higher than non-Maori (307). In the 2010/2011 New Zealand Health Survey, 5.5% of NZ adults and 30% of individuals aged 75+ had a diagnosis of ischaemic heart disease with the figures for stroke being 1.8% and 8% respectively (308). Recognizing factors that modify this risk is essential to alleviate the burden of disease.

Calcium intake either dietary or by calcium supplements may be one such modifiable mechanism to reduce CVD burden. Although L.K. Baldauf identified the presence of calcium in diseased aortas in 1906 (309), it wasn't until the middle of last century that studies linking the association of calcium with CVD began to emerge. Indeed, in 1936 Haythorn *et al.* made the profound statement, *" The affinity of the tissues of the aorta in advanced years for calcium is greater than that of the circulating blood"* (310). This, along with their own radiological findings, lead Elkeles and Berlin to conclude in 1957 that "*a good deal of calcium and phosphorus, no longer utilised by the bones, is deposited in the aorta… I therefore suggest that a relationship exists between the predominance of calcified atheroma in women aged more than 60 and postmenopausal osteoporosis"* (311). In spite of this, two threads have continued side by side in seemingly direct contrast with each-other: dieticians and those interested in bone health favouring an increase in calcium intake, with recommended intakes for those of greatest age second only to those through puberty; whereas those of the cardiac specialties introducing calcium antagonists for the treatment of hypertension and angina.

Although an adequate *dietary calcium* intake may be beneficial, over the last 20 years there has been a steady crescendo in the concern that calcium supplements may be harmful to cardiovascular health. Calcium supplements increase serum calcium (312-314) and elevations in serum calcium, even at the upper end of normal, are associated with CVD (315).

"If calcium is accumulating in the arteries while leaking from the skeleton, can we assume that supplemental calcium goes selectively to bone?"

Linda L. Demer

A Skeleton in the Atherosclerosis Closet

Circulation 1995 (92); 2029-2032

1.6.1. Dietary calcium and cardiovascular risk.

In the bone community, high calcium intakes from any source have been considered safe: "*calcium intakes up to at least 2500 mg are safe for virtually all patients*" (316). And, given that calcium supplements and dietary calcium produced similar skeletal effects, there was a belief that a distinction regarding calcium source was not required (317). The findings of Chapuy *et al.* contributed to the impetus of these recommendations (48,284), but the generalisability of this study has been called into question (301). Broadening our interest to the effects of calcium on the cardiovascular system challenges the belief that high calcium intakes are safe and that the body handles dietary calcium and calcium supplements similarly. Calcium supplements increase serum calcium (292,312,314,318-326). This contrasts with dietary calcium intakes which do not appear to correlate with serum calcium (327,328). The magnitude of the effect of calcium supplements on serum calcium may be modulated by a number of factors but dosing in the morning or the evening does not appear to be one of them (323). A higher peak in serum calcium is observed with a larger supplement dose (323), possibly by Vitamin D co-administration (319) (329), and when dietary calcium intake is

restricted (320,322).

1.6.2. Dietary calcium and cardiovascular risk.

Suggestions that calcium intake may be beneficial to cardiovascular health initially came in the form of ecological studies, cohort and case control studies analysing an association with hardness of drinking water (330-333). However, the findings were inconsistent with inherent weaknesses, and drinking water supplied a relatively low amount of calcium compared with dietary sources (334).

Prospective observational studies of dietary calcium intake and its association with cardiovascular disease risk have been summarized by Wang *et al.* (335). Their meta-analysis compared the highest to the lowest quintile of dietary calcium intake across several studies with a pooled relative risk (RR) of 0.92 (95% confidence interval CI, 0.80, 1.07) for any coronary artery disease (six studies with two studies contributing both male and female arms) and RR 0.86 (95% CI 0.69, 1.06) for any stroke (7 studies with 1 study contributing both male and female arms) suggesting that dietary calcium intake is neither harmful nor beneficial to cardiovascular health. However, some studies have reported a non-linear, U-shaped interaction and if this is the case, simply comparing the highest quantile (Wang *et al.* range of 665 – >1245 mg/day) with the lowest quantile (Wang *et al.* range of 233 – 585 mg/day) may be an insufficient comparison especially if both the highest and lowest quintiles are in aspects of the curve conferring similar risk.

Larsson *et al*. (336) report a U-shaped association between dietary calcium intake and stroke incidence and stroke-related mortality. They performed a large dose-response meta-analysis of 11 observational prospective studies that were conducted between 1996 and 2012 in European, Asian and American populations with an age range of 34-99 years. Combined, these studies accounted for 9,095 strokes in 403,831 individuals with all studies adjusting for

age and smoking status, and most studies adjusting for alcohol consumption, body mass index, physical activity, history of diabetes, history of hypertension and other nutrient intakes. They found an inverse non-linear relationship between calcium intake and stroke risk at low to moderate baseline calcium intakes with a weaker association at higher intakes. From their curve, the dose conferring the lowest relative risk was approximately 1000 mg/day with intakes of 700-1700 mg/day lying at or below a relative risk of 1.0. Interestingly, the downward slope observed between doses of 200-700 mg/day was steeper than the upward slope in doses greater than 1700 mg/day suggesting that small increases in calcium at low dietary intakes rapidly reduce stroke risk. In populations with low baseline calcium intake (<700 mg/day), the risk of stroke was reduced by 18% after increasing calcium intake by 300 mg/day. Additionally, in Asian populations who had inherently low calcium intakes at baseline (5 studies with average intake of 469 mg/day across all studies, range 406-591 mg/day), they observed a 22% decrease in the risk of stroke for every 300 mg/day increase in calcium intake. A reduction in risk was not observed in studies with an average calcium intake >700 mg/day

Six studies provided information regarding stroke subtype. Where dietary calcium intake was <700 mg/day, a 300 mg/day increase resulted in reduced risk for both ischemic stroke (RR 0.84; 95% confidence interval 0.77-0.93) and intra-cerebral haemorrhage (RR 0.80; 95% CI 0.68-0.95) but not for subarachnoid haemorrhage (RR 0.83; 95% CI 0.55-1.26).

The investigators of the Swedish mammography cohort report a U-shaped association between dietary calcium intake and CVD specific mortality (337). In this study, 61,433 women were followed prospectively for 19 years and all-cause mortality as well as cause specific CVD mortality was reported (337) with 3,862 and 1,932 deaths from CVD and ischaemic heart disease (IHD) respectively. Across the four primary outcome measures of all cause, CVD, IHD and stroke mortality, the calcium intake associated with an adjusted hazard

ratio (HR) of ≤1 was approximately 700-1200 mg/day and was preserved throughout the four curves. From the point of the lowest risk, the correlation between increases in calcium intake and death differed between cardiovascular subtypes with the steepest incline observed in deaths from IHD and the most gradual in deaths from stroke which are consistent with the findings of Larsson *et al*.

Xiao *et al*. also report a U-shaped association for CVD deaths but only for men and only when considering total calcium intake from both dietary and supplemental sources (338). In their prospective study of 23,652 predominantly males (83.5%) of mean age 40.8 years, Kwak *et al.* found in a population with low dietary calcium intake (213.6-499.2 mg/day) that dietary calcium was not associated with an increased risk for coronary artery calcification score (339).

Tables 1.6.1-1.6.3 summarize prospective studies considering dietary calcium intake and risk of CVD death, CHD death and incident IHD respectively. They depict the highest and lowest quintiles of dietary calcium intake in each study and the calcium intake associated with the lowest risk. Figure 1.6.1-1.6.3 depict the mean dietary calcium intake or range associated with the lowest risk for CVD death, CHD death, and incident IHD. Although many of these studies have findings consistent with a U-shaped association, the intake range associated with the lowest risk is still broad. The reason for this may relate to the intrinsic weaknesses of cohort studies but may also represent the physiological adaptations that occur to maintain homeostasis.

In 2016, Chung *et al.* published an updated systematic review and meta-analysis on calcium intake and cardiovascular disease risk (340). Unfortunately, this has only created more confusion (341,342). Some of their analyses have compared dietary and total intakes of >1000 mg/day with intakes <1000 mg/day. If a U-shaped relationship exists for the impact of calcium on cardiovascular risk then this comparison is inappropriate. Furthermore, if this U-

shaped relationship shows a shallower gradient at higher calcium intakes, comparing this point of the curve with low intakes that have a steeper gradient will certainly give the appearance of a reduced risk.

Table 1.6.1 Prospective studies on dietary calcium intake and risk of death from cardiovascular disease. For studies that analysed male and female data separately, these are depicted by two sets of results in each box. Results pertaining to men are positioned above those for women.

STUDY	Participants	*p* for trend	events (*n*)	Lowest quantile (mg/day)	Highest quantile (mg/day)	Intake of lowest risk (mg/day)
Michaelsson *et al*., 2013 Swedish Mammography Cohort 19yr follow-up	Women 61,433 Mean age 53.9 years	NA	3,862	<600	≥1,400	600-999
Kaluza *et al*., 2010 Swedish men cohort 10yr follow-up	Men 23,366 Age: 45-79 years	0.064	819	<1,230	≥1,599	≥1599
Li *et al*., 2012 EPIC Heidelberg - Germany 11yr follow-up	Men and Women 23,980 Age: 35-64 years	0.62	267	513	1,130	513
Khan *et al.*, 2015 Melbourne Collaborative Cohort Study 13.3yr follow-up	Men 17,045 Women 24,469 Age 40-69 years	0.28	557	641	1076	899
Umesawa *et al*., 2006 JACC study Japan 9.6yr follow-up	Men 21,068 Women 32,319 Age: 40-79 years	0.95 0.14	685 644	250 266	665 667	536 545
Xiao *et al.*, 2013 NIH-AARP study, USA 12yr follow-up	Men 219,059 Women 169,170 Age: 50-71 years	0.08 0.37	7,904 3,874	478 408	1,247 1,101	616/898[a] 648
Van der Vijver *et al*., 1992 Dutch Civil Servants Study 28yr follow-up	Men 1,340 Women 1,265 Age: 40-65 years	NA	NA	≤585 ≤445	>1,245 >850	>1,245 >850

STUDY	Participants	*p* for trend	events (*n*)	Lowest quantile (mg/day)	Highest quantile (mg/day)	Dose of lowest risk (mg/day)
Van Hemelrijck *et al.*, 2013 NHANES III, USA. 8.2yr follow-up	Men and Women 18,714 Age: >17 years	0.025	855 1,015	<500 <500	>1,300 >1,300	>1,300 <500-1,000[a]
Dai *et al.*, 2013 The Shanghai Men's Health study 4-8yr follow-up The Shanghai Women's Health study 10-14yr follow-up	Men 61,414 age 40-74 Women 73,232 age 40-70	0.21 0.57	800 1147	<408 <408	≥800 ≥600	600- ≤800 <408

[a]2 quantiles with similarly low risk

Table 1.6.2 Prospective studies on dietary calcium intake and risk of death from coronary heart disease. For studies that analysed male and female data separately, these are depicted by two sets of results in each box. Results pertaining to men are positioned above those for women.

STUDY	Participants	p for trend	events (*n*)	Lowest quantile (mg/day)	Highest quantile (mg/day)	Lowest risk (mg/day)
Michaelsson *et al*., 2013 Swedish Mammography Cohort 19yr follow-up	Women 61,433 Mean age 53.9	NA	1932	<600	≥1400	600-999
Bostick *et al*., 1999 Iowa Women's Health Study, USA 8yr follow-up	Women 34,486 Aged 55-69 years	0.14	387	<543	>1110	>1110
Khan *et al.*,2015 Melbourne Collaborative Cohort Study 13.3y follow-up	Men 17,045 Women 24,469 Age 40-69 years	0.48	157	641	1076	1076
Umesawa et al., 2006 JACC study Japan 9.6yr follow-up	Men 21,068 Woman 32,319 Aged 40-79 years	0.43 0.50	148 116	250 266	665 667	363 545
Xiao *et al.*, 2013 NIH-AARP study, USA 12yr follow-up	Men 219,059 Women 169,170 Aged 50-71 years	0.04 0.93	6282 2777	478 408	1247 1101	616/898[a] 648
Van der Vijver *et al*., 1992 Dutch Civil Servants Study 28yr follow-up	Men 1,340 Women 1,265 Aged 40-65 years	NA	NA	≤585 ≤445	>1245 >850	≤585 >850
Van Hemelrijck *et al*., 2013 NHANES III, USA. 8.2yr follow-up	Men and Women 18,714 Aged >17 years	0.525	510 496	<500 <500	>1300 >1300	>1300 >1300
Dai *et al.*,2013 Shanghai Men's Health Study 4-8yr follow-up Shanghai Women's Health Study 10-14yr follow-up	Men 61,414 Age 40-74 Women 73,232 Age 40-70	0.12 0.94	395 511	<408 <408	≥800 ≥600	≥800 Similar across quartiles

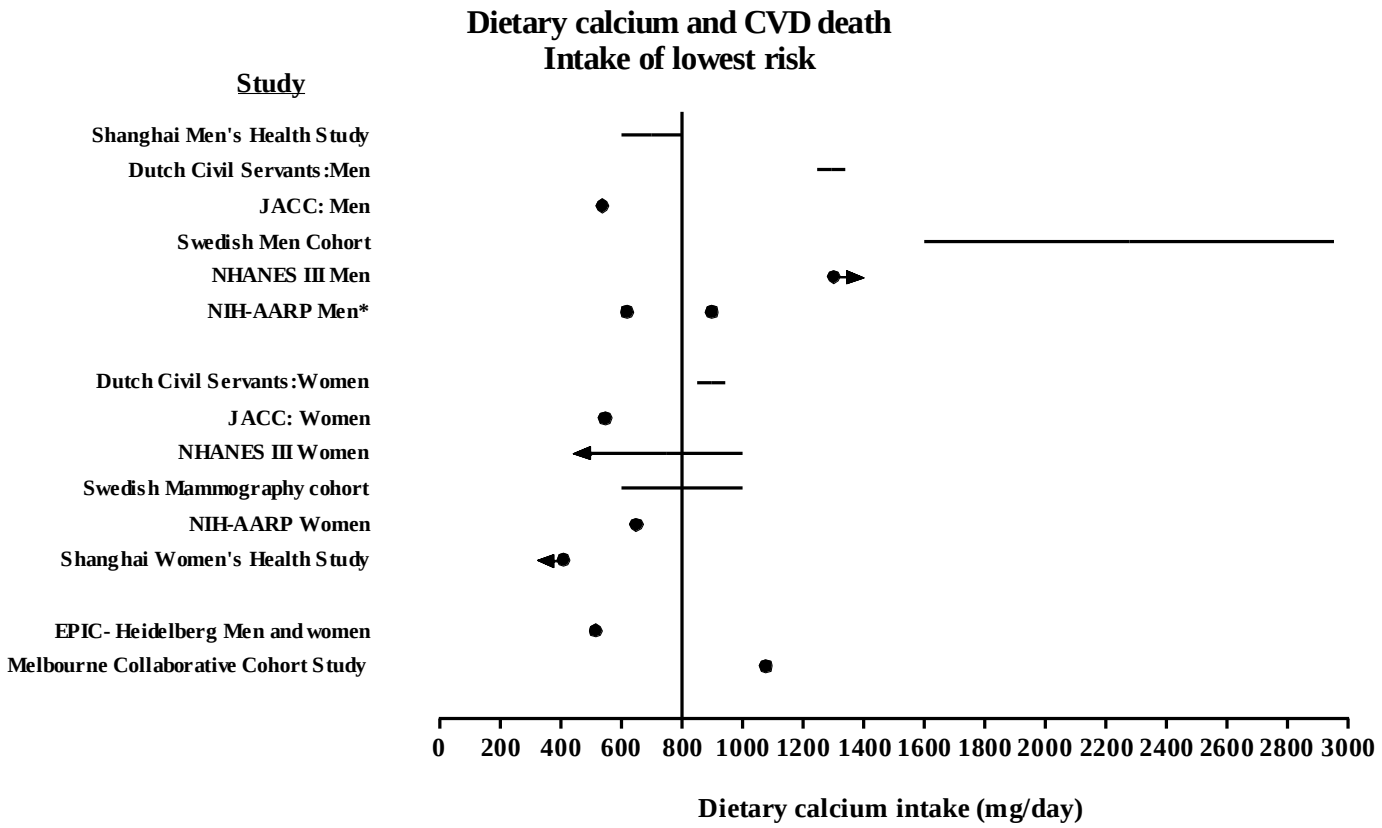
[a]2 quantiles with similarly low risk

Table 1.6.3 Prospective studies on dietary calcium intake and risk of incident coronary heart disease. For studies that analysed male and female data separately, these are depicted by two sets of results in each box. Results pertaining to men are positioned above those for women.

STUDY	Participants	p for trend	events (*n*)	Lowest quantile (mg/day)	Highest quantile (mg/day)	Lowest risk (mg/day)
Al-Delaimy et al., 2003 MIH-Follow up study, USA 12yr follow-up	Men 39,800 Aged 40-75 years	0.11	1458	497	1190	880
Umesawa et al., 2008 JPHC study Japan 13y follow-up	Men and Women 41,526 Aged 40-59 years	0.60	322	233	753	439/753[a]
Li et al., 2012 EPIC Heidelberg, Germany 11yr follow-up	Men and Women 23,980 Aged 35-64 years	0.22	354	513	1130	820
Khan *et al.*,2015 Melbourne Collaborative Cohort Study 13.3y follow-up	Men 17,045 Women 24,469 Age 40-69 years	0.036	1827	641	1076	1076

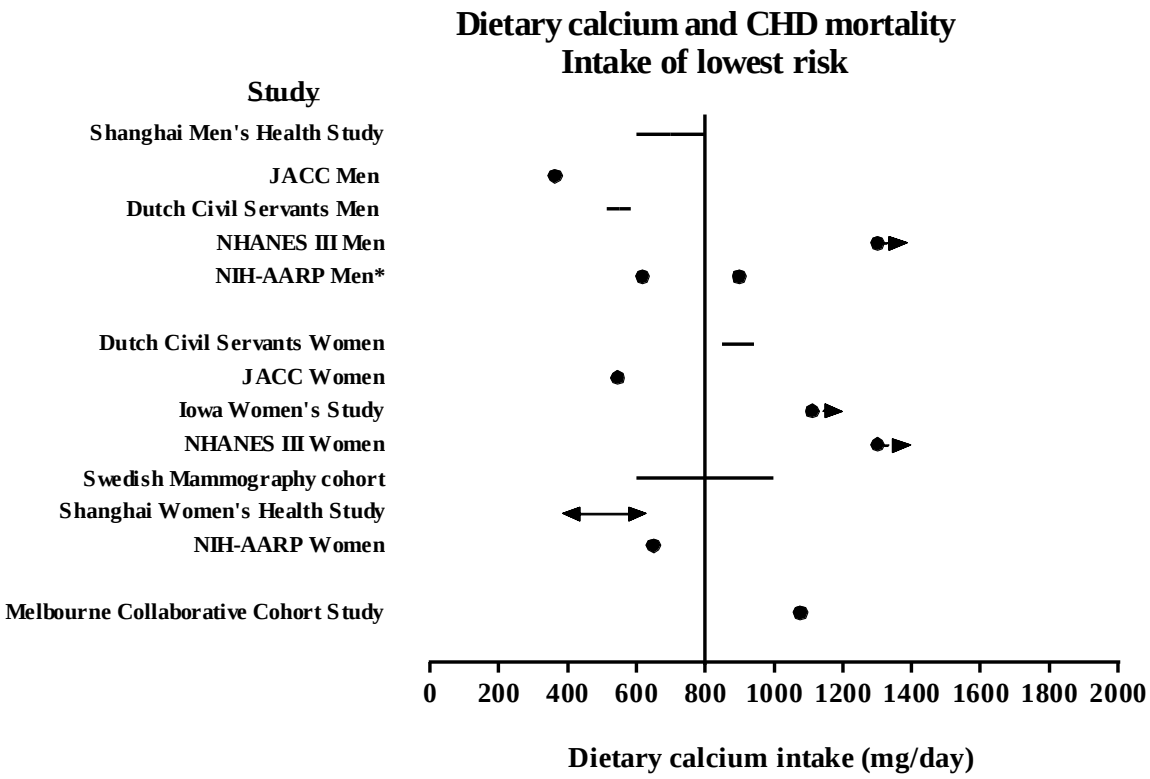
[a]2 quantiles with similarly low risk

Figure 1.6.1 Prospective studies on dietary calcium intake and risk of death from cardiovascular disease. Studies reporting a mean intake are represented by a dot. Studies reporting an intake range are represented by horizontal lines. Arrows are used when maximum or minimum dietary intakes have not been stated.



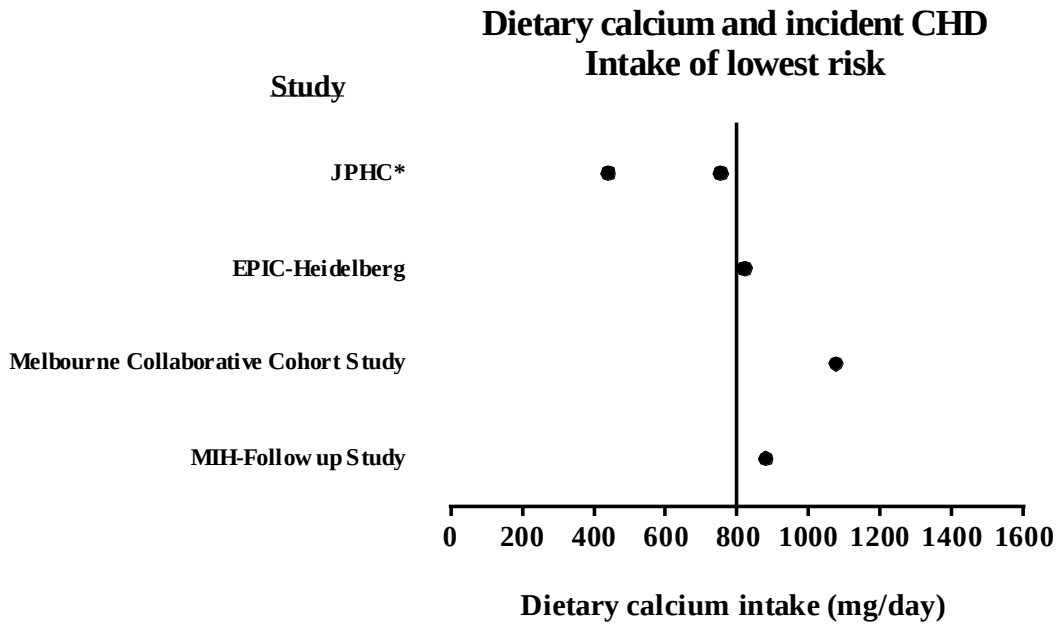
*2 quantiles with similarly low risk

Figure 1.6.2 Prospective studies on dietary calcium intake and risk of death from coronary heart disease. Studies reporting a mean intake are represented by a dot. Studies reporting an intake range are represented by horizontal lines. Arrows are used when maximum or minimum dietary intakes have not been stated.



*2 quantiles with similarly low risk

Figure 1.6.3 Prospective studies on dietary calcium intake and risk of incident coronary heart disease (CHD). Studies reporting a mean intake are represented by a dot. Studies reporting an intake range are represented by horizontal lines. Arrows are used when maximum or minimum dietary intakes have not been stated.



*2 quantiles with similarly low risk

1.6.3. Serum calcium and cardiovascular risk.

The homeostatic mechanisms that govern serum calcium do so within a narrow range, indicating that deviations outside this range may have adverse consequences. The cardiovascular effects of primary hyperparathyroidism provide some evidence to validate this notion. Primary hyperparathyroidism is associated with hypertension, left ventricular hypertrophy, heart failure and calcific disease culminating in increased mortality due to myocardial infarction and cardiovascular disease (343,344). Both increases in circulating PTH and hypercalcaemia contribute to this risk. Although hypercalcaemia is thought to play a dominant role in the calcific deposits observed throughout the vasculature (343), PTH itself is associated with an increased risk for coronary heart disease (345).

Perturbations of serum calcium occur with age. In 12,865 men between the ages of 25 and 97 years, Jorde *et al.* observed a decline in serum calcium from a mean value of 2.41 mmol/L in the youngest age group to 2.36 mmol/L in the oldest (346). In women, age produced interesting changes in serum calcium. From the ages of 25 through to 39 years, mean serum calcium was 2.35 mmol/L. In the next decade this rose to 2.36 mmol/L, followed by a further increase of 0.03 mmol/L during the ages of 50-59 years, and peaking at 2.40 mmol/L through the next 2 decades of 60-79 years (346). This increase in serum calcium is consistent with the dramatic bone wasting that occurs through the perimenopausal transition, and the presumed secondary effects of reduced intestinal calcium absorption and serum 25-hydroxyvitamin D (347-350).

Concerningly, serum calcium within the normal range is associated with adverse cardiovascular effects. Many prospective cohort studies have identified an association between serum calcium and fatal and non-fatal cardiovascular events (351-356). This has been ratified in a meta-analysis showing a hazard ratio of 1.08 (95% CI 1.04, 1.3) for the risk

of myocardial infarction or coronary heart disease per standard deviation of serum calcium (approximately 0.1 mmol/L) (315,341). Importantly, the effect of serum calcium on cardiovascular events is both direct and indirect, mediated in part by cardiovascular risk factors such as systolic hypertension (315,357,358). The positive correlation between serum calcium and coronary artery calcification score is also important (339). Serum calcium is also associated with conventional and non-conventional components of the metabolic syndrome. Serum calcium is inversely related to the beneficial adipokine adiponectin; and positively correlated with triglycerides, fasting glucose, fasting insulin and measures of oxidative stress (359).

Thus, menopause signifies a transition to increased cardiovascular risk governed by an increase in serum calcium, a temporary increase in serum PTH (350), as well as the direct consequences of oestrogen loss (see section 1.2).

1.6.4. Calcium supplements and cardiovascular risk.

In light of the beneficial effect calcium supplements had on circulating lipids in postmenopausal women, calcium supplements were expected to lower cardiovascular risk (360). It was under this premise that the initial analysis of the effect of calcium supplements on cardiovascular risk, a secondary analysis of a randomised controlled trial of calcium supplementation in healthy postmenopausal women, was undertaken (288). The findings however, were in direct contrast to this: calcium supplements increased cardiovascular risk. Given the marked deviation from ambient thought, it was necessary to confirm these results. Unfortunately, the subsequent meta-analysis failed to disprove this finding. In this meta-analysis of randomised controlled trials of calcium supplements where cardiovascular endpoints were collected in studies primarily designed to assess bone-related and in some instances colorectal adenoma endpoints, Bolland *et al.* found that calcium supplements taken

in isolation increased the risk of myocardial infarction by 31% (p = 0.035) where patient level data were available (5 studies; n = 8,151; women = 6,345), and by 27% (p = 0.038) in the analysis of trial level data (11 studies; n = 11,921) (289). When women from the Women's Health Initiative Calcium and Vitamin D trial (WHI CaD) (361,362) who were not taking personal calcium supplements at baseline (n = 16,718) were added to the available patient level data of the meta-analysis (n = 24,869), calcium with or without vitamin D increased the risk of myocardial infarction by 26% (p = 0.005)(363). Stroke risk was also increased (hazard ratio HR 1.19; 95% CI 1.02, 1.39; p = 0.03) as was the composite endpoint of either myocardial infarction or stroke (HR 1.17; 95% CI 1.05, 1.31; p = 0.005).

An important question is whether participants of the WHI CaD study, who were taking non-protocol calcium supplements at randomisation, should or should not be included in analyses of the effects of calcium supplements on cardiovascular endpoints. The original publication for the Women's Health Initiative investigators reported that calcium and vitamin D supplementation had a null effect on the risk for coronary or cerebrovascular disease (362). However, 54% of women involved in the trial were taking non-protocol calcium supplements at randomisation. Bolland *et al.* found that personal use of calcium supplements at randomisation significantly influenced the effect treatment allocation had on cardiovascular outcomes. Given the placebo group is no longer a true placebo but effectively a combination of placebo and a lower dose calcium supplemented group, it is appropriate to exclude these from analyses.

Lewis *et al.* have performed a further meta-analysis of the effects of calcium supplementation of coronary heart disease (364). Although in supplementary data these investigators have performed analyses excluding women in the WHI CaD who used non-protocol calcium supplements, for their main analysis they have included these women. The conclusion from

their analysis indicates a null effect of calcium supplements on cardiovascular risk. Bolland *et al.* outline how these results do not contradict their 2011 meta-analysis but validate them. Through the assiduous appraisal of the analyses performed by Lewis *et al.*, the Auckland group identified a number of factors that contributed to the difference in outcome: the choice of a composite endpoint as their primary endpoint; the inappropriate inclusion of a trial by Larsen *et al.*; the inability to include patient data from one study; and the decision to exclude men from their analyses (365). A response that Lewis *et al.* find disingenuous (366).
As mentioned, in order to inform a joint position statement from the National Osteoporosis Foundation and American Society for Preventive Cardiology, Chung *et al.* have performed a further re-analysis of available data using innovative techniques allowing them to assess dose response metaregressions of prospective cohort studies, for the effect of calcium intake on cardiovascular outcomes, excluding some randomised controlled trials that were of poor design (340). For their analyses of the effect of dietary calcium and calcium supplements, they included 4 randomised-controlled trials, 1 case-control study, and 26 cohort studies. Consistent with the findings of Lewis *et al.* these investigators did not find evidence for an increased risk for cardiovascular events with calcium supplements with or without vitamin D. Concerns regarding study design have been raised (342).

Reid *et al.* aptly describe the emergence of multiple re-analyses of the same data as *"bewildering to anyone not intimately familiar with these data."* (341) In this publication, the Auckland group carefully addresses the discrepancies between their own results and the subsequent re-analyses of the data.

1.6.5. Conclusion.

An adequate dietary calcium is necessary to provide the resource for normal cell function. However, the effect of calcium supplements on the body is dissimilar to that of dietary

calcium, with the former increasing serum calcium, a situation that has adverse consequences on cardiovascular health. Whether studies reach significance for the effect of calcium supplements on the risk of cardiovascular disease is dependent on what data is included in analyses. The careful, measured and substantiated arguments presented by the Auckland group are compelling. Considered together, these indicate that the practice of prescribing calcium supplements to postmenopausal women as a substitute for, or to supplement, dietary calcium intake, should be re-evaluated.

Whether the recommended calcium intakes for postmenopausal women are excessive is not the subject of this thesis. However, the value of the recommended intake for dietary calcium should consider the following, prospective cohort studies indicate that high calcium intakes (>1000 mg/day) in postmenopausal women provide no additional benefit to cardiovascular health compared with adequate intakes (~800 mg/day); meta-analyses find high dietary calcium (and calcium supplements) ineffective for the prevention of osteoporotic fractures; and finally, the circulation of postmenopausal women is not in a state of calcium lack, in fact bone wasting raises serum calcium which homeostatically decreases intestinal calcium absorption and serum vitamin D. Furthermore, an inappropriately high recommended calcium intake may create anxiety amongst postmenopausal women, who may feel pressured and confused about how they might achieve this.

1.7. Vascular biology.

Vascular smooth muscle cells provide the elastic recoil properties of blood vessels which allow them to store up to 50% of the left ventricular stroke volume (367), enabling blood to continually circulate while the heart is in diastole. They are responsible for the vessel tone with their contraction and relaxation mediating vessel vasoconstriction and vasodilation respectively; and for the peripheral resistance which the heart, in systole, must overcome in order to effectively supply the peripheral tissues with circulating blood. Vascular smooth muscle cells secrete an extracellular matrix which provides vessel stability through matrix-cell interactions, and serves as a storage reservoir of growth factors that are released during wall injury.

In spite of the histologically indistinguishable appearance of vascular smooth muscle cells throughout the vasculature, different anatomical regions have diverse embryological origins which render them distinct in their response to varying morphological cues. That is, smooth muscle cells from different lineages do not have the same response to identical influences (368). Another unique feature is a retained capacity for phenotypic transformation, an ability that allows vascular smooth muscle cells to transform into a proliferative, synthetic and secretory cell type which is necessary for the repair of injured vessels. Once the integrity of the vessel is restored, they trans-differentiate into the quiescent contractile phenotype.

1.7.1. Embryology and origins of vascular smooth muscle cells.

Vascular smooth muscle cells have heterogenous, non-overlapping origins from at least seven embryonic sites (368). This means that vascular smooth muscle cells from different vessels, and sometimes within the same vessel, are derived from different embryological origins (Table 1). In conjunction with this, their response to various differentiation signals is lineage-dependent. A downside of this phenomenon is that vascular smooth muscle cells from one

lineage have a limited capacity to rescue the loss of cells from another lineage as they do not respond in an appropriate site-specific manner to local cues (369). The boundaries between cells from different origins are sharply demarcated. These boundaries can be the basis of disease, with dissections in Marfan's syndrome arising at the junction between vascular smooth muscle cells originating from the neural crest and secondary heart field (370). A further interesting observation is the interdependency of the different embryologically derived tissues that form the heart, proximal vasculature and surrounding tissue. As an example of this, cardiac neural crest cells support the growth of the myocardium, and if the migration of these cells to the region of the developing heart is impeded, cardiomyocyte morphogenesis is disrupted (369,371).

1.7.1.1. Cardiac neural crest.

Cardiac neural crest cells are derived from a subpopulation of neural crest cells between the otic placode and the third somite. From here they migrate ventrally through the pharyngeal region to coalesce around the preformed endothelial-lined lumens of the pharyngeal arteries, differentiating to form the medial layer of the 3rd, 4th and 6th pharyngeal arch arteries, before remodelling into their final form. Cardiac neural crest cells form the aortic arch and arch vessels, the smooth muscle cells of the subaortic and subpulmonary region, the aortopulmonary septum thus dividing the truncus arteriosus into the pulmonary trunk and ascending aorta, and the ductus arteriosus (372). They also form part of the ventricular septum and contribute to the formation of the heart. During migration, cardiac neural crest cells interact with each-other, the extracellular matrix and cells of the pharyngeal arches through which they traverse, as well as the endothelium of the developing vasculature. Defects in pre-migratory proliferation, epithelial to mesenchymal transformation, migration and differentiation, in addition to changes in the surrounding milieu with which they interact, can produce defects in mice, chicks and zebrafish that resemble congenital abnormalities

found in humans. These include persistent truncus arteriosus, double outlet right ventricle, an interrupted aorta, double aortic arch, left-sided origin of the right subclavian artery, and the cardiac congenital malformations observed in DiGeorge syndrome, CHARGE syndrome and Alagille syndrome (373).

While in the neural crest, cardiac neural crest cells must undergo proliferative expansion which appears to be mediated by the transcription factor Pax3 (*splotch*) and the downstream signalling protein Wnt-1 (371). Thereafter they undergo epithelial to mesenchymal transformation with optimization of adhesion status culminating in the differential expression of various cadherins. The migrating cells depend on N-cadherin downregulation for migration, but require upregulation for outflow tract remodelling. Interactions between N-cadherin and connexin 43 on migrating cells, is also important for the regulation of polarized cell movement (374).

During migration, cardiac neural crest cells come into contact with cells whose ligand expression directs migration and encourages differentiation. The downstream effects of the semaphorins, the transcription factor Tbx1, and endothelin-1 signalling appear to be important in directing the stream of migrating cells to their appropriate fate.

The semaphorins are a family of intercellular signalling proteins known for their attractive and repulsive actions in axonal guidance during development (375). The class III semaphorins, Sema-3C, and the class VI semaphorins, Sema-6A and -6B, appear to be important in normal patterning of the cardiac outflow tract, arch and arch vessels (376,377). The semaphorin receptors Plexin-A2 and Plexin-D1, are expressed in the migrating cell population along with their obligatory protein component, neuropilin-1. Plexin-A2 and Plexin D1 receptors mediate repulsive and attractive responses respectively. Sema-6A and -6B in the neural crest repulse the cardiac neural crest cells, which are attracted by fibronectin 1 along

their migratory path (378). Sema-3C expression in the cardiac outflow tract serves as an attractant for the migrating cells to their final destination.

Cell migration to populate the 4th pharyngeal arch artery is dependent on the action of the T-box transcription factor Tbx1. The pharyngeal surface ectoderm, which forms the outer surface of the pharyngeal arches, expresses Tbx1 which interacts with the gastrulation brain homeobox 2 (Gbx2) protein to upregulate the formation of the secreted ligand Slit. Interaction of Slit with the transmembrane protein receptor Robo, found on migrating cardiac neural crest cells, restricts and directs their migratory path towards the 4th pharyngeal arch (379).

Endothelin, a potent vasoconstrictor, is expressed by the endothelium of the pharyngeal arch arteries and appears to function in an inhibitory capacity. In addition to the deficient development of the aortic arch manifesting as aortic arch interruption or hypoplasia, endothelin-1-null mice also show persistence of the 1st and 2nd pharyngeal arch arteries giving rise to extra common carotid vessels. This phenotype is reproduced in the endothelin type-A receptor knockout mice but not in endothelin type-B receptor knockout mice. It appears that endothelin-1, expressed by the resident endothelium of the 1st and 2nd pharyngeal arches, interacts with endothelin type-A receptor on migrating cardiac neural crest cells, discouraging them from populating these vessels, instead directing them caudally to the 4th, 5th and 6th pharyngeal arches (380).

Post-migration, cardiac neural crest cells undergo differentiation into vascular smooth muscle cells, a process that started at the time of neural crest departure (377). The transcription factor, myocardin-related transcription factor B (MRTF-B), plays a critical role in the appropriate patterning of neural crest derived vascular smooth muscle cells. Serum response factor is a constitutive nuclear transcription factor that plays a critical role in the transduction

of cytoskeletal signals to the nucleus and thereby regulating the expression of genes encoding cytoskeletal proteins. It does this by binding to CArG boxes (an A/T rich sequence CCWWWWWWG) in DNA transcriptional regulatory elements. MRTF-B is an essential cofactor to serum response factor-dependent transcription of cytoskeletal elements necessary in vascular smooth muscle differentiation (381-383).

Notch signalling also stimulates differentiation of post-migratory cardiac neural crest cells into vascular smooth muscle cells. Although notch ligands of the jagged and delta-like proteins are both implicated in notch receptor signalling, jagged-1 appears to be the ligand involved in the differentiating cardiac neural crest cells. After the intracellular portion of the notch receptor is released and translocates to the nucleus, it forms a transcriptional complex with several transcriptional co-activators. Hairy and enhancer of split (HES)-related transcription factors (HRT) 1-3 appear to be the target genes responsible for vascular smooth muscle cell differentiation. It is unclear whether the jagged-1 ligand present on the surface of the endothelium of the pharyngeal arch arteries, or that expressed by the neural crest-derived tissue enveloping them, is necessary for this notch-related differentiation (384).

Other mechanisms are able to influence Notch signalling. The histone deacetylase Hdac3 regulates transcription through both chromatin-mediated (epigenetic) transcriptional repression and direct deacetylation of key transcriptional regulators. In the differentiating cardiac neural crest cell, Hdac3 is necessary for the upregulation of jagged1 that occurs post-notch signalling (385). Fibronectin, acting through the integrin α5β1 on cardiac neural crest cells is another mechanism (378).

1.7.1.2. Proepicardium.

The proepicardial organ is a transitory structure originating from the secondary heart field mesenchyme, which forms at the venous pole of the heart tube. These cells form the

epicardium and from here, by a process of epithelial to mesenchymal transformation, form the smooth muscle cells and fibroblasts of the coronary arteries; the cardiac fibroblasts; the atrioventricular cushions, which will form the heart septa; the atrioventricular valves and the fibrous skeleton of the heart. They also regulate the proliferation and differentiation of the underlying myocardium (386).

From the proepicardial organ, these mesenchymal cells migrate over the surface of the heart tube, enclosing the developing structure with epicardium. This process is facilitated by myocardial vascular cell adhesion molecule-1, α4 integrin expressed by the migrating proepicardial cells, and Tbx5. Wilms tumour suppressor WT-1 is also implicated in formation of the epicardium (387). Concurrent with epithelial to mesenchymal transformation, proepicardial cells invade the underlying myocardium in a defined spatiotemporal fashion that is governed by inhibitory signals originating in the myocardium. This process is influenced by Notch signalling, with downstream effects on endothelial-derived PDGF-B (388) and TGFβ (389) (390); canonical Wnt signalling (391); fibroblast growth factor signalling (392); as well as transcription factors (393) and adhesion molecules (387).

1.7.1.3. Secondary heart field.

The progenitor cells that differentiate to form the heart arise from the splanchnic lateral plate mesoderm. Here, the primary and secondary heart fields are contiguous, separated by spatiotemporal patterning. Even after this separation, there is further temporal division of the secondary heart field which supplies consecutive waves of cells to the developing heart. The primary heart field forms the initial bilateral endocardial tubes which, through embryonic folding, form a single midline tube with the venous pole inflow located caudally and the arterial outflow pole located rostrally. At the completion of heart formation, the primary heart field forms the left ventricle. The cells of the secondary heart field form the right ventricular

and outflow tract myocardium, the atria and the atrioventricular canals, the sinus venosus, contributing vascular smooth muscle cells to the aortic root and the pulmonary trunk (370). Therefore, the secondary heart field contributes both to the venous and arterial poles of the endocardial tube with the cells of the primary heart field in the middle.

Control of spatial patterning is required to determine both anterior-posterior and left-right asymmetry in the developing heart. Retinoic acid limits the posterior extent of heart field by repressing the fibroblast growth factor 8-mediated generation of cardiac progenitors (394).

Left-right patterning is initiated at the time of the primitive streak, where ciliated epithelium at the distal tip generate a predominant leftward fluid flow. This results in the formation of the nodal protein and subsequent nodal signalling. Through its transcriptional effector, Foxh1, nodal upregulates its own protein expression, that of its antagonist Lefty2, and the protein Pitx2c which is responsible for outflow tract rotation and patterning of the atria and pulmonary veins. Sonic hedgehog signalling also influences nodal production and therefore left-right patterning (395).

The transcriptional factor Nkx2.5, is essential for cardiac formation (396). It has multiple cis regulatory elements which generate at least three distinct transcripts, enabling spatiotemporal control of Nkx2.5 expression (397). Through the inhibition of Smad1, Nkx2.5 inhibits the differentiating function of bone morphogenetic proteins therefore maintaining the secondary heart field in a proliferative state while the first heart field differentiates (398). It is also important in maintaining the identity of ventricular and atrial cardiomyocytes (399).

Both fibroblast growth factor 8 (Fgf8) and sonic hedgehog maintain proliferation of progenitors in the secondary heart field, for subsequent deployment. Fgf8-null mice display double outlet right ventricle and persistent truncus arteriosus as a result of a lack of sufficient progenitors contributing cells to the outflow tract myocardium (398,400). Sonic hedgehog

supports the developing myocardium, and the smooth muscle cell population of the outflow tract in the arterial pole (398).

Differentiation occurs through the downregulation of canonical Wnt signalling enabling bone morphogenetic protein activity to induce the cardiomyocyte genetic profile. Activity in the non-canonical Wnt pathway, mediated through Wnt 5a; and stimulation by bone morphogenetic protein 2 and 4, lead to the differentiation of secondary heart field progenitors. Ligands for the canonical Wnt pathway, Wnt3a and Wnt8, inhibit differentiation. Accordingly, Dickkopf-1, the Wnt ligand antagonist; and Notch, which negatively regulates β-catenin; both promote differentiation (398).

1.7.1.4. Somites.

The embryological origin of the adult descending thoracic aorta is remarkable as the initial populating cells are no longer present in the adult aorta (216). Endothelial cells are initially derived from the splanchnic lateral mesoderm. As the paired aortae migrate medially, they acquire cells from the dorsally positioned somite mesoderm which incorporate into the dorsal surface of the aorta (401). Within the aorta, the ventrally-positioned splanchnic lateral mesoderm-derived cells gradually form haematopoietic clusters which enter the foetal circulation to populate the haematopoietic organs, leaving no trace of their former presence within the aortic wall (401-405). The adult descending thoracic aorta is therefore, comprised of cells originating in the somite or paraxial mesoderm.

Notch signalling is important for the attraction of somite-derived vascular smooth muscle cells (401,406). Jagged1 ligand on resident endothelial cells interacts with Notch3 on migrating vascular smooth muscle cells initiation the smooth muscle program. EphrinB2 is likely to act downstream of Notch signalling (407). The importance of Notch is shown in mice deficient in both Notch2 and Notch3. Here, the dorsal aorta is formed normally but later

collapses because of the lack of resident smooth muscle cells (408). Notch signalling permits stimulatory action of Wnt signalling driving the smooth muscle cell program preventing somite-derived progenitors from becoming endothelial cells (401,406).

Vascular smooth muscle cells of the coronary arteries and adult aorta have diverse embryological origins. The media of the coronary arteries is derived from a single origin, the proepicardial organ. The adult aorta, however, has diverse origins. Vascular smooth muscle cells of the subaortic valve and subpulmonary valve regions originate from the secondary heart field, from here, the cardiac neural crest cells supply cells which form the outflow tract, aortic arch and arch vessels. Somite-derived cells populate the descending thoracic aorta. Although not discussed here, the arterial supply to the gut is also derived from the lateral splanchnic mesoderm and occurs in a similar fashion to the formation of coronary arteries (409).

Table 1.7.1: Embryological origins of the vascular smooth muscle cells that form the proximal and cardiac arterial blood vessels.

Embryological origin of vascular smooth muscle cells					
	Ectoderm	**Mesoderm**			
		Splanchnic lateral plate			**Axial/Somite**
Arterial vessel	**Neural Crest**	**Secondary Heart field**	**Proepicardial organ**		
Coronary arteries			√		
Sub aortic and sub pulmonary vessel		√			
Ascending aorta	√				
Aortic arch	√				
Carotid vessels	√				
Descending aorta					√
Intercostal vessels					√
Abdominal aorta				√	

1.7.2. Vascular smooth muscle cell phenotypic modulation.

Vascular smooth muscle cells guard the structural and functional integrity of blood vessels. They integrate signals from multiple origins to preserve the circulation of blood around the body by rearranging their cytoskeleton, altering vascular tone, and elaborating an extracellular matrix. In preserving the delivery of blood to the periphery they must partake in vessel repair, minimizing the ability of the underlying matrix to stimulate thrombosis, and maintaining the connectivity necessary for coordinated function. To fulfil this task, vascular smooth muscle cells have the ability to modulate their phenotype, switching from a contractile state with a low proliferative rate, to one signified by one or a combination of the following: loss of contractile protein expression (also referred to as de-differentiation), migration, proliferation and synthesis.

In the cell, phenotypic modulation is regulated by a few key pathways which integrate information received through humoral interactions; cytoskeletal elements, modified by mechanical influences; and by cell-matrix and cell-cell interactions. The combinatorial effect of the stimulatory and inhibitory effectors, fine-tunes the cell response producing the final observed phenotype. It is important to note the interdependency of the endothelial and vascular smooth muscle cells. Mechanical influences like shear stress, flow pattern and cyclical stretch from pulsatile blood flow stimulate the release of endothelial-derived hormonal signals which influence vascular smooth muscle cell phenotype (410).

1.7.2.1. Serum response factor/Myocardin-related transcription factor.

The interaction of the transcriptional factor, serum response factor (SRF) and its smooth muscle-restricted co-factors, myocardin-related transcription factors; and the modification of this relationship, exemplify the transcriptional and post-translational mechanisms that regulate smooth muscle cell phenotypic modulation.

SRF is a ubiquitous transcription factor that, through a MADs-box domain, binds to a CArG box sequence within *cis*-regulatory elements of DNA, to initiate gene transcription. SRF is able to initiate the transcription of many vascular smooth muscle cell contractile proteins through co-activation with myocardin-related transcription factors (MRTF). There are three known MRTF: myocardin, MRTF-A and MRTF-B, and although they share some common functionality, differences in their amino acid sequences denote lineage-restricted activity. In vascular smooth muscle cells, unlike other cell types, these transcriptional cofactors are found in the nucleus (411). Mechanisms that promote a change from the quiescent contractile phenotype destabilize the MRTF releasing them from the nucleus.

Ternary complex factors of the E twenty-six (Ets) domain family, Elk-1, SAP-1 and SAP-2, compete against MRTF for SRF binding thereby preventing the transcription of contractile proteins (412). Instead, they promote the synthetic phenotype by upregulating *c-fos* transcription therefore inducing cell proliferation and survival (413). Phosphorylation of Elk-1 by MAP kinases increases its affinity for SRF binding (414).

Some transcription factors and repressors influence vascular smooth muscle cell phenotype by inducing epigenetic modifications. Epigenetic regulation is defined as the mechanisms that alter heritable cell phenotype without alterations in DNA sequence. One such mechanism is the modification of histone tails by methylation or acetylation which affects the accessibility of transcription factors to the *cis* binding elements on DNA. Histone acetylation, by histone acetyl transferases, creates a permissive environment for SRF-dependent transcription of contractile proteins. Alternatively, inhibition of histone acetyl transferases or expression of histone deacetylases promotes de-differentiation. Myocardin can interact with histone complexes in two ways. Firstly, by interaction with histone tails myocardin is able to stabilize SRF-myocardin binding to the CArG box; and secondly it is able to recruit histone acetyl

transferase, favouring the permissive orientation of histones, promoting the contractile phenotype (415,416). Conversely, the transcriptional repressor PRISM, promotes the synthetic phenotype by many mechanisms, one of which is through interaction with histone deacetylases (415,417). Similarly, kruppel like factor 4, induced by platelet-derived growth factor-BB (PDGF-BB) stimulation, inhibits myocardin and promotes the synthetic phenotype (411).

Ligand-generated effects can promote the action of SRF and its cofactor MRTF thereby inducing the contractile complement of genes, or inhibit SRF-driven transcription and therefore alter phenotype. Typically, growth factors such as PDGF, endothelial growth factor and insulin-like growth factors promote the synthetic phenotype. Conversely, transforming growth factor β (TGF-β) and bone morphogenetic proteins promote MRTF-SRF activity through the activation of Smads. Angiotensin II can both promote and repress the contractile phenotype depending on the intracellular signalling pathway. Similarly, Notch has dual actions: working cooperatively with TGF-β it promotes the contractile phenotype, but through its activation of the transcriptional inhibitor hairy-related transcription factor 1, can antagonize the expression of differentiation markers (416).

Cytoskeletal actin polymerization also induces transcription of the contractile complement. Rho activation induces actin polymerization by stimulating the incorporation of globulin-actin (G-actin) into filamentous actin (F-actin). In non-smooth muscle cells, MRTF is bound to G-actin and the incorporation of G-actin into F-actin releases MRTF to translocate into the nucleus (418). However, given that MRTFs are observed exclusively in the nucleus of smooth muscle cells, the mechanism by which Rho signals induce the transcription of the contractile complement is yet to be identified in vascular smooth muscle cells but may be through direct or indirect effects of the transcription factors nuclear factor of activated T-cells

(NFAT) and/or GATA-6 (419-421) or through the regulation of the SRF subcellular location (422).

Finally, many non-coding RNAs (miRNA) regulate vascular smooth muscle cell phenotype by altering the activity of transcription factors, coactivators and corepressors. For example, miR-145 is able to repress Klf4 and miR-143 represses Elk-1. Together, these miRNA promote differentiation while concurrently repressing proliferation (416).

1.7.2.2. Cell to cell interactions.

Vascular smooth muscle cells interact with each-other and with endothelial cells at myoendothelial junctions. These myoendothelial junctions are cellular extensions which extend through the internal elastic lamina bringing the two cell types in apposition to each-other. Here, gap junctions form, permitting the transfer of information and coordinating local function (423).

Gap junctions are formed by transmembrane gap junction proteins called connexins, which consist of two hemichannels contained within the membrane of neighbouring cells. Connexins (Cx) 37, 40, 43 and 45 are found in vascular smooth muscle cells with connexin 45 found here exclusively (416). In arterial vascular smooth muscle cells, connexins are implicated in the maintenance of the contractile phenotype, and modulation to the synthetic phenotype. In coronary arteries, the contractile smooth muscle cell phenotype is promoted by interaction with endothelium through a Cx43-Cx40 heterotypic gap junction (424). This is observed in static co-cultures as well as cultures submitted to a shear stress of 12 dyn/cm^2, which is thought to be physiological. However, reduced shear stress (5 dyn/cm^2) resulted in a simultaneous downregulation of endothelial Cx40, and an upregulation of endothelial Cx43 resulting in a homotypic Cx43-Cx43 gap junction which mediated smooth muscle cell de-differentiation. Consistent with these findings is the enrichment of Cx43 in endothelial cells

of bovine coronary arteries at sites near a bifurcation (425), sites commensurate with increased atherogenicity.

1.7.2.3. Cell to matrix interactions.

Arterial walls consist of three layers. From the vessel lumen they are the tunica intima, which is comprised of a single layer of endothelial cells; the tunica media, which varies according to site and is bound on the intimal side by the internal elastic lamina, and on the adventitial surface by the external elastic lamina; and the tunica adventitia, which is mainly composed of collagen, elastin and fibroblasts. The large elastic arteries of the aorta, brachiocephalic, common carotid and subclavian arteries have an extremely elastic tunica media comprised of multiple concentric sheets of elastin and collagenous tissue, with relatively few smooth muscle fibres. The tunica media of the smaller muscular arteries, the radial, femoral, coronary and cerebral arteries, is comprised of a thick layer of circumferentially arranged smooth muscle cells enabling calibre changes in the blood vessel that regulate the flow of blood to the vascular beds they supply (426).

The extracellular matrix is an indispensable multifaceted structure. Its components provide the tensile and elastic strength necessary in a cyclically pressured environment. In the event of injury, the extracellular matrix is equipped with growth factors that, along with protein exposure, stimulate a change of functional requirement to the resident cellular populations. These then adopt a migratory, synthetic and secretory phenotype restoring homeostasis. To support these functional requirements of the blood vessel wall, the extracellular matrix contains a plethora of proteins which bind the different operational components together. Because of their modular complexity, these components also act as multi-purpose molecules.

The subendothelial basement membrane not only provides structural support to the endothelium, but also a physical barrier to cellular penetration. Its major components are

collagens type IV and XVIII, laminins, nidogens, perlecan and the blood glycoprotein von Willebrand factor. Collagen type IV and laminins form independent networks within the basement membrane and these are connected to each-other by nidogens. Perlecan exemplifies the glycoprotein family of heparan sulfate proteoglycans to which it belongs. In the vessel wall these are the cell membrane-bound syndecans and GPI (glycosylphosphatidylinositol)-anchored proteoglycans, and within the extracellular matrix: perlecan and type XVIII collagen. Their multiple functional modules allow them to interact with a number of extracellular matrix proteins: growth factors, antithrombin III, chemokines, fibronectin, laminins, and act as coreceptors (427,428). Accordingly, perlecan has an important function in stabilizing the basement membrane and can also protect laminin against proteolytic degradation (429,430).

As mentioned, the anatomical functions of the various arterial segments define the components of its extracellular matrix. The tunica media of the large elastic arteries contain multiple concentric layers of elastin fibres, which endow the vessel wall with compliance and elastic recoil; and collagens I and III which are not elastically deformable and therefore set the limits of vessel expansion (429). Tropoelastin is secreted by vascular smooth muscle cells, endothelial cells and fibroblasts. It undergoes a process of self-aggregation, referred to as coacervation, attached to cell surface integrins; is then deposited on microfibrillar scaffolds, made up of fibrillins and fibulins, which promotes further coacervation; and then forms intra and intermolecular crosslinks after the enzymatic processing by lysyl oxidase, recruited by fibulin-5 (431). Fibulins 1, 2 and 5 interact with elastin, microfibrillar components and cell bound integrins indicating a role in cell to matrix adhesion (432). Like fibulins, the glycoprotein fibronectin is able to bind to a number of extracellular matrix constituents including cell surface integrins, collagen and heparan sulfate proteoglycans (433).

These components of the vessel wall denote the interactions of the vascular smooth muscle cell in its contractile, differentiated state. Deviations away from this stimulate a phenotype change towards the fully de-differentiated state of synthesis, migration and proliferation.

Matrix metalloproteinases degrade the matrix through proteolytic cleavage of many matrix components including collagens I, II, III, IV, V, VII, X; fibronectin and elastin (434). In doing so, they relieve vascular smooth muscle cells from the physical constraints on cell movement; and release degradation products and matrix-sequestered growth factors which together promote the synthetic phenotype. Monomeric collagen type I, found in degraded matrix, promotes de-differentiation (416,435). Similarly, the degradation of elastin into elastin-derived peptides induces de-differentiation, migration and proliferation (436). Degradation products can also escalate the response to injury by recruiting and inducing the activity of monocytes (430).

TGFβ (437) and some bone morphogenetic proteins (438) stimulate the differentiated, contractile vascular smooth muscle cell phenotype. A number of growth factors (427), liberated from the extracellular matrix and secreted from cells, downregulate TGFβ. Fibroblast growth factors (439) and vascular endothelial growth factor work in this way inducing the SMC synthetic phenotype (440). However, vascular endothelial growth factor does this indirectly through stimulating endothelial secretion of fibroblast growth factors. Platelet-derived growth factor induces vascular smooth muscle cell proliferation directly through Akt and ERK 1/2 pathways (441).

1.8. Vascular calcification.

1.8.1. Calcification occurs in the vessel intima and media.

Atherosclerosis is an inflammatory vascular process of the subintima that can progress to cause luminal narrowing or occlusion leading to the acute coronary syndrome, myocardial infarction, stroke and transient ischaemic attacks. Although patients with familial hypercholesterolaemia have accelerated atherosclerotic lesions and can manifest disease as early as the first decade of life (442,443), uncomplicated atherosclerosis progresses slowly with age associated with the "inflammaging" phenomenon, that is that aging is associated with a chronic inflammatory status (444,445). Atherosclerosis begins as a fatty streak, the process builds with increasing cellular complexity and organization terminating in a plaque that often contains a core containing necrotic debris, cells and lipids. Initially this process is contained under a fibrous cap rich in vascular smooth muscle cells (VSMC) but disruption of this cap through cell death can lead to plaque rupture and manifest clinical disease (446).

Atherosclerosis is a complex process initially dominated by the innate immune system but later involving B cells and T cells. It involves chronic inflammation at sites of disturbed laminar flow as is seen at anatomical branching sites, sites of endothelial dysfunction as may be caused by cigarette smoking, and at sites of structural alteration such as non-confluent luminal elastin layers or proteoglycan exposure (447).

Cholesterol is transported in the blood by low-density lipoproteins containing apolipoprotein B100. At sites of vascular intimal injury apolipoprotein B100 – low-density lipoprotein (LDL) complexes attach to extracellular matrix where LDL is susceptible to oxidation by reactive oxygen species or by enzymes such as myeloperoxidase and lipoxygenase which are released from activated macrophages. The now oxidized LDL then induces the expression of leucocyte adhesion molecules and chemokine release from endothelial cells and, together

with platelet chemokines, recruit leucocytes and potentiate the process (448). Recruited monocytes are activated in the subintimal layer accumulating lipids and becoming foam cells which form the initial fatty streak. Synthetic VSMC are recruited contributing to luminal narrowing through proliferation and production of an extracellular matrix. Calcification occurs within the atherosclerotic plaque triggered by the atherogenic milieu (449).

Whereas inflammation secondary to age, flow dynamics and endothelial dysfunction are major drivers in atherosclerosis-associated intimal calcification, medial calcification seen in chronic kidney disease (CKD) is directed by mineral imbalance with increases in serum phosphate and calcium playing important roles. In addition to this, calcification is also increased by secondary physiological changes (hyperparathyroidism) and CKD treatment (vitamin D and warfarin) with the uraemic milieu also accelerating age-associated atherosclerotic changes. Patients with diabetes mellitus also display increased prevalence of intimal and medial calcification. Here hyperglycaemia results in an increase in advanced glycation end-products which induce oxidative stress and inflammation and also increase calcification of VSMC (450). In CKD and diabetes mellitus, vascular calcification is observed at a younger age with progressive lesions and poorer prognosis (451). It is important to note that the two locations of vascular calcification are not mutually exclusive and share many of the same risk factors.

1.8.2. Association with cardiovascular disease.

As mentioned, vascular calcification is associated with cardiovascular disease where calcification in one vascular bed provides site-specific risk, but is also associated with manifest disease at other anatomical sites (451). In the coronary arteries, calcification results in reduced vascular compliance, abnormal vasomotor responses and impaired myocardial perfusion. It is also associated with overall atherosclerotic plaque burden (452,453), with an

increased risk of cardiac events in asymptomatic individuals (454) and has an increased mortality risk (455). It predicts coronary artery disease risk in addition to scoring systems based on risk factors alone, such as the Framingham risk score (455). Additionally, it modulates outcomes following revascularization procedures, with calcification leading to technical difficulties in coronary artery bypass grafting, late saphenous graft failures and poorer percutaneous intervention outcomes.

Interestingly, increasing severity of coronary calcification may provide survival benefit. Increased plaque density scores, a measure of calcification extent, showed an inverse relationship for both coronary heart disease and cardiovascular disease events in a study using data from the Multi-Ethnic Study of Atherosclerosis trial (456). Furthermore, individuals on statin treatment showed higher density scores and lower event rates which may explain their treatment benefit. Some have suggested that it is the number of mineral interfaces that introduces plaque instability, with a homogenous plaque conferring stability compared with plaques that have multiple spots of calcification (457).

Calcium deposition on cardiac valves, both native and prosthetic, is a cause for valve failure and is associated with all-cause mortality (458-460). Aortic valve calcification may lead to calcific aortic stenosis which results in left ventricular hypertrophy, left ventricular failure and sudden cardiac death. It is also associated with coronary events (461).

Calcification of the main arterial tree – the ascending, descending and abdominal aorta - is also associated with significant cardiovascular morbidity. Stiffening of the arteries causes loss of the Windkessel effect, an elastic buffering system whereby the aortic wall is able to store about 50% of the left ventricular stroke volume which it delivers peripherally during diastole (367). A loss of this effect results in increased pulse pressure, elevated systolic pressure and ultimately loss of perfusion pressure. This culminates in an increased risk for

myocardial infarction, stroke, peripheral vascular disease and heart failure preceded by left ventricular hypertrophy.

In CKD vascular calcification is often far-reaching. Calcification occurs in the vascular beds previously mentioned but a calcific process that occurs predominantly in this patient population is calciphylaxis also known as calcific uraemic arteriolopathy. Here, systemic medial calcification results in widespread small vessel occlusion and subsequent necrosis of skin and fat. Mortality is extremely high, with one study reporting 71% mortality at 1 year (462,463).

1.8.3. What causes calcification? Passive versus active theories.

Previously thought to be an unavoidable degenerative process, it is now accepted that vascular calcification shares many similarities to skeletal bone deposition, albeit a pathological response to injury. Calcium deposition in vessels is the result of a complex interplay of local and distant calcification inhibitors and stimuli, the extracellular milieu, and local cells that integrate the available information either avoiding calcium deposition or when inhibitory mechanisms are exhausted, producing calcifying matrix vesicles. It is important to understand that tissue fluids, for example blood, urine and saliva, are supersaturated with respect to hydroxyapatite (464-466). This is substantiated by the finding that irrespective of their calcium or phosphate concentration, serum samples have similar saturation indexes with respect to amorphous calcium phosphate (465) and that normal vessels do not calcify in experimental conditions of increased calcium and or phosphate (467). This supersaturation is important to maintain bone and to provide nucleating substrates. However, in order to prevent mineralization of soft tissues serum must be stable and remain unmineralized. These biofluids use pH and chaperoning proteins to prevent mineral precipitation. We now know that posner clusters, small calcium phosphate pre-nucleational complexes, circulate in the serum

chaperoned by Fetuin-A and albumin (468,469). Some have interpreted the increased calcium-phosphate product associated with vascular calcification that is observed in CKD as causal, lending weight to the theory that vascular calcification is a passive process (470). That is, that as the calcium-phosphate product increases mineral precipitation will passively occur. This is likely a surrogate marker for the loss of serum chaperones. An extension of this passive theory is that inactivating gene mutations of calcification inhibitors result in calcium deposition.

The argument favouring an active cause for vascular calcification comes from the observation that VSMC and other cell types of mesenchymal origin (pericytes, fibroblasts) undergo an osteochondrocytic phenotypic transformation induced by a number of triggers (471). These stimuli decrease the transcription of smooth muscle cell specific genes and converge on *Runx2, Sox 9* and *Msx2* initiating osteochondrocytic transition (472-474). With evidence of endochondral and intramembranous ossification in blood vessels, some have suggested that vascular calcification should instead be referred to as vascular ossification (471).

1.8.4. Pathogenesis.

Vascular calcification is a complex process which may result from a single catastrophic loss of vital inhibitors, or the accumulation of multiple persistent triggers involving a number of organ systems whose interconnectedness transcends the body's ability to avert extra-skeletal hydroxyapatite formation. Stimuli induce one or more of the following, initiating vascular calcification, 1) production of mineralizing-competent matrix vesicles released from VSMC, 2) apoptosis of VSMC, 3) a phenotypic transition of VSMC to an osteochondrocytic lineage or 4) absent or reduced presence of calcification inhibitors. Thereafter, crosstalk acts to stoke mineralization.

Many cells types are involved in vascular calcification, both locally in the vessel wall and at distant sites resulting in autocrine, paracrine and endocrine signalling. Peripherally, the kidneys, bone and parathyroid glands are involved in phosphate and calcium homeostasis along with the intestine. The liver is involved in cholesterol processing and formation of the calcification inhibitors, pyrophosphate and Fetuin-A. Fetuin-A is taken up by VSMC and packaged into matrix vesicles (475,476). Circulating macrophages become foam cells and secrete cytokines which drive VSMC inflammation. Unsurprisingly, all cells of the vascular wall are involved in the ectopic calcification process.

1.8.4.1. Signalling pathways.

Bone morphogenetic proteins (BMPs) and transforming growth factor-β (TGF-β), members of the TGF-β superfamily, are implicated as extracellular ligands involved in vascular calcification, having both proinflammatory effects and driving the metamorphosis of VSMC to an osteogenic lineage and the ensuing calcification (477-481). Although other extracellular ligands are involved to fine tune cellular response, signalling through these two groups alone results in considerable functional complexity given their ability to signal through multiple combinations of TGF-β and BMP receptors (482). Receptors transmit to the nucleus through both smad and non-smad pathways (MAPK, PI3K/Akt, PKC) which also interrelate (482,483). These signals are further regulated externally by co-receptors (endoglin and betaglycan) which potentiate signalling, and internally by inhibitory Smad (Smad 6/7) which stimulate receptor degradation and maintain the VSMC phenotype. Extracellular soluble receptor antagonists, like sclerostin, prevent receptor signalling (484,485), whereas other antagonists, like matrix gla protein and Fetuin A, bind to TGF-β or BMPs directly (486,487). A decoy receptor has also been identified lacking an intracellular domain therefore sequestering BMPs (488).

1.8.4.2. Matrix vesicles and apoptosis.

VSMC apoptosis and the formation of matrix vesicles (MV) play a key role in vascular calcification (489,490). An understanding of their role in physiological bone formation is helpful when considering their role in ectopic calcification.

Osteoblasts first secrete an extracellular matrix or osteoid which is able to conform to the area that will be occupied by bone. MV are the "matchstick" which when set alight with calcium phosphate crystals, form the crystal ignition for the epitaxial growth of bone within osteoid along the collagen scaffold. Ossification occurs in two phases. Phase one involves MV budding from the parent cell. Phosphate and calcium transporters increase the intra-vesicular concentration of each respective ion and along with AnxA5 and phosphatidylserine form the nucleational core complex. Crystal formation appears to be initially in the form of amorphous calcium phosphate changing to octocalcium and finally hydroxyapatite. Phase two begins with hydroxyapatite perforating the MV membrane exposing itself to the extracellular fluid after which epitaxial crystal proliferation is governed by the interplay of extracellular conditions: available phosphate provided by membrane phosphatases, and the relative concentration of the calcium-binding matrix proteins bone sialoprotein, osteonectin, osteocalcin and collagen type I and II (491).

MV are extracellular membrane invested particles that are selectively enriched with various components of their parent cell. Released from chondrocytes, osteoblasts and odontoblasts by polarized budding of the outer plasma membrane, they contain phosphatases, lipids, annexins, non-collagenous calcium-binding proteins and Type III sodium-dependent phosphate transporters Pit 1 and Pit 2. They also contain BMPs and matrix metalloproteinases with the latter resorbing the cartilaginous matrix permitting vascular invasion (492). Phosphatases provide substrate for crystal formation: phosphoethanolamine/phosphocholine

phosphatase, as well as the phosphate transporters Pit 1 and 2, plays a role in the initiating phase of crystal formation whereas alkaline phosphatase, and enzymes targeting adenosine monophosphate, adenine triphosphate, and pyrophosphate are important in the propagating phase of crystal growth (493). Importantly, ectonucleotide pyrophosphatase/phosphodiesterase 1 generates pyrophosphate which at low concentrations (<1 mM) provides a substrate for pyrophosphatase and alkaline phosphatase in the generation of inorganic phosphate, but at higher concentrations (>1 mM) inhibits mineralization (493).

Although MV produced by VSMCs bear many similarities to those of skeletal origin (annexins are present to facilitate budding off from the parent membrane and selective assembly of proteins) there are many differences which stem from their opposing function (494). In bone, MV serve as the initiator of controlled mineralization and therefore their constituents are balanced in favour of ossification. However, in the vasculature, MV are released in a bid to maintain cell viability by expelling excess calcium and phosphate and therefore their components are geared, at least initially, against mineralization. An example of this is that VSMC-derived MV have reduced alkaline phosphatase activity and contain calcification inhibitors (494). (Please see “1.8.4.4 Calcium” and “1.8.4.6 Calcification inhibitors” for further elaboration)

Some have felt that MVs are actually apoptotic bodies with MV release from chondrocytes a step in the process of programmed cell death. However, others have shown that MVs and apoptotic bodies differ in their ability to concentrate calcium and this along with the spatial differences in the appearance of MV and the apoptotic chondrocytes in the growth plate support the notion that although similar, the two are different (495).

MVs are selectively enriched in the acidic calcium binding lipids phosphatidylserine and phosphatidic acid. Phosphatidylserine is particularly important in mineralization. It displays

membrane asymmetry detectable only on the cytoplasmic surface in viable cells. It has a high affinity for calcium and, along with phosphate and Annexin A5 , serves as the nucleational core complex within the MV and on vesicle surface which is the first site of crystal formation (494,496,497). In apoptosis, cell membrane asymmetry is lost and phosphatidylserine is translocated to the external surface of the cell, signalling availability for phagocytosis. Here, the high affinity of Annexin A5 for phosphatidylserine is utilized as a marker of apoptosis (498).

Annexins are multifunctional proteins that interact with the phospholipid membrane in a reversible and calcium-dependent manner. They appear to be important in membrane organization and trafficking and can form lateral assemblies which act as membrane scaffolds - a possible explanation as to how MV are able to selectively enrich themselves with various proteins. They are important in facilitating ion fluxes across the cell membrane and can act as calcium channels and calcium and pH sensors (499). A clear understanding of their role in health and disease has been impeded by the apparent built-in redundancies, with upregulation of some annexins to compensate for the loss of others. However, some studies found no such upregulation in specified knockouts leading to the conclusion that there may be structural and functional redundancies within the annexin family to compensate for the knockout. This is the case for the Annexin A6 knockout mice, in which there is no clear phenotype. That said, some helpful insights can be derived from *in vitro* work. MV secreted by calcifying bovine VSMCs show increased Annexin A2 and Annexin A6 content, as well as increased calcium uptake and increased ability to calcify on collagen type I (490). Conversely, when the annexin calcium channel was inhibited, calcium uptake and calcification were impeded.

Other proteins play important roles in the regulation of mineralization. Bone matrix proteins bone sialoprotein, osteonectin and osteocalcin (also known as bone Gla protein) are calcium-

binding proteins, with bone sialoprotein appearing to be bone promoting whereas osteonectin and osteocalcin appear to inhibit mineral growth.

Apoptosis is programmed cell death of which there are three known pathways. Firstly, the extrinsic pathway is transmembrane receptor-mediated with a cytoplasmic death domain. Examples include Fas and TNF-α. Secondly, cytotoxic T lymphocytes secrete a transmembrane pore-forming molecule in the Perforin/granzyme pathway allowing serine proteases Granzyme A and Granzyme B to enter the cell and activate pro-caspases. Finally, the Intrinsic pathway is non-receptor-mediated and following various stimuli leads to the opening of the mitochondrial permeability transition pore with subsequent erosion of the transmembrane potential. This is largely governed by the Bcl-2 family of proteins which are pro- or anti-apoptotic, determining whether a cell commits to or aborts the apoptotic programme (500).

In atherosclerotic plaques VSMC apoptosis it can be detrimental (501). In addition to providing a nidus for mineral formation, apoptosis causes plaque instability by thinning of the fibrous cap and adds to the inappropriate vasomotor response (502-504). For unknown reasons, apoptosis within atherosclerosis leads to inefficient clearing of debris by both VSMCs and recruited macrophages. The persistence of apoptotic bodies and cells is thought to provide the ongoing stimulus for inflammation. Hydroxyapatite crystals also induce apoptosis and contribute to growing mineralization by inducing cell damage as well as inducing BMP-2 signalling (505,506).

In VSMC, those factors that stimulate apoptosis and increase MV production also result in calcium deposition (507).

1.8.4.3. Phosphate.

Inorganic phosphate is required for cellular function and skeletal mineralization, with 80-90% stored in the bone as hydroxyapatite. Body phosphate is maintained through intestinal absorption by two pathways: paracellular and active absorption through the type II sodium dependent phosphate transporters, uptake and release from bone stores, and regulated renal reabsorption predominantly through type II sodium dependent transporters. Crosstalk between these mechanisms is achieved through PTH, vitamin D and other phosphatonins including fibroblast growth factor-23 (508-510).

That phosphate might play a role in vascular calcification was highlighted by the observation that patients with CKD have elevated phosphate levels which in turn are associated with an increased mortality risk (511-513). This prompted searches in populations without renal dysfunction identifying the association of serum phosphate with cardiovascular disease risk in the general population. (353,356,514-516). Further indications implicating phosphate handling in ectopic calcification were that deficiency in the *Klotho* gene, a coreceptor for the actions of the phosphaturic hormone fibroblast growth factor-23 in the kidney, leads to arteriosclerosis as well as ectopic calcification (517,518), and that transgenic overexpression of tissue non-specific alkaline phosphatase (TNAP also alkaline phosphatase), the enzyme responsible for hydrolysing the mineralization inhibitor pyrophosphate into the pro-calcific inorganic phosphate, in VSMC as well as endothelial cells leads to vascular calcification (519-521). We now know from human and rodent studies that phosphate contributes to vascular calcification in the following ways:

1. Regulation of SMC phenotype with initiation of osteogenic transdifferentiation (522-525).

2. Increases TNAP, hydrolysing pyrophosphate producing phosphate. TNAP also increases the expression of BMP-2 in VSMC and alters the phosphorylation status of osteopontin negating its protective effect. Therefore upregulation of TNAP has a greater effect than just the production of Pi alone (526-529).
3. Induces VSMC apoptosis (530-532).
4. Induces elastin degradation which promotes vascular calcification (533).
5. Increases MV release and subsequent extracellular mineralization (489).

Unlike handling in the intestine and kidneys, phosphate uptake in VSMC is mediated by the type III sodium dependent phosphate transporter PiT-1 encoded by the *Scl20a1* gene in humans (524,534,535). A recent study suggests that PiT-2, the second member of the type III sodium dependent phosphate transporters, prevents vascular calcification in mice fed a high phosphate diet (536,537).

1.8.4.4. Calcium.

VSMC respond to elevated levels of extracellular calcium by attempting to pre-empt the cascading events that lead to vascular calcification. Initially, signalling through the calcium-sensing receptor stimulates an increase in the inhibitor matrix gla protein and off-loading of cell calcium through upregulated release of matrix vesicles and downregulation of intra-vesicular alkaline phosphatase activity (467,538-540). If the calcium stress is transitory these mechanisms suffice to prevent mineralization. However, with on-going calcium stress the cell is unable to keep up with the demands of high, competent inhibitor-laden, matrix vesicle output producing uncarboxylated matrix gla protein, and Fetuin-A-deficient matrix vesicles loaded with activated matrix metalloprotein-2 and therefore mineralization occurs (539).

Elevated extracellular calcium promotes calcification in other ways. Calcium promotes the translocation of Anxa6 from the cytosol to the plasma membrane and along with

phosphatidylserine presents these for cell surface expression and inclusion within matrix vesicles (539). This, along with the increased apoptosis observed with increased extracellular calcium, provides nucleational sites. Finally, calcium increases PiT-1 expression which mediates the phenotypic transition of VSMC to an osteogenic lineage, enabling phosphate-induced pathways of mineralization (541).

1.8.4.5. Matrix metalloproteinases.

Matrix metalloproteinases (MMP) are zinc metallo-endopeptidases that promote tissue growth and remodelling by selective proteolytic degradation. They comprise eight structural groups with five subgroups based on substrate specificity, although there is built in overlap. Their activity is modulated by gene expression, post-translational modifications, activation of the proenzyme and inhibition by tissue inhibitor of metalloproteinases (TIMPs). Many MMPs are secreted as a proenzyme and are activated extracellularly, but some are activated within the cell and/or on the cell surface. MMP-2 is of the "gelatinase" subgroup and is known to cleave collagens I, II, III, IV, V, VII, X, elastin, fibronectin, and activate TGF-β, TNF-α and IL-Iβ. MMP-2 is activated on the cell surface by binding with TIMP-2 and relieved of its pro sequence by membrane-type metalloproteinase I, although another isoform is active within the cell. MMP-2 can also be activated by MMP-1, MMP-7, thrombin and activated protein C (434,542,543).

MMP-2 is able to effect vascular calcification in two ways: through the degradation of elastin as well as other proteins of the extracellular matrix, and through cell-mediated mechanisms. MMP-2 can stimulate an osteogenic phenotypic switch of the VSMC from within and without the cell. From within, proteolysis of calponin-I, a protein involved with contractile machinery as well as a phenotype marker, results in a phenotypic switch (544). Extracellular MMP-2 is able to cleave elastin and release elastin-derived peptides. Elastin degradation is

associated with increased vascular calcification (545,546) and this can be abrogated by the MMP inhibitor doxycycline (544,547). Elastin-derived peptide binds to its receptor on the VSMC surface, elastin laminin receptor, and in conjunction with cleavage of type IV collagen which tethers the cell to the basement membrane and the induced upregulation of BMP-2 expression, MMP-2 instigates osteogenic lineage reprogramming (548-550).

1.8.4.6. Calcification inhibitors.

The formation of bone requires the provision of ample substrate provided in calcium and phosphate to support its function. The task of the extracellular milieu is to provide this substrate in the form of posner complexes without mineralization occurring within the circulation or extracellular space, a task it is well endowed to perform in the presence of circulating calcification inhibitors. Additionally, local cell populations require an additional inhibitor supply when under stress. Matrix gla protein, pyrophosphate and Fetuin-A are three such inhibitors.

1.8.4.6.1. Matrix gla protein.

Matrix Gla protein (MGP) prevents calcification by blockade of the osteo-inductive and perhaps also the pro-apoptotic properties of bone morphogenetic protein-2 (BMP-2) and, in conjunction with Fetuin A, by complexing with and sequestering calcium and phosphate within MV to prevent calcification (551-553).

MGP is a small protein (14 kDa) that is expressed by a number of cell types including chondrocytes, VSMC, fibroblasts and endothelial cells. It undergoes two types of post-translational modifications with five of its nine glutamate residues undergoing vitamin K-dependent γ-carboxylation (abbreviated to “gla”) enabling the protein to bind calcium ions.

The second modification is a serine phosphorylation which may be important for secretion (554,555).

In bone formation MGP plays a vital role in chondrocyte viability and function with biphasic expression during chondrocyte maturation (556). In cultures of hypertrophic chondrocytes MGP controls the degree of mineralization with overexpression resulting in reduced mineralization and interference of the formation of gla residues leading to extensive mineralization (557).

In vascular health MGP prevents vascular calcification by sequestering calcium and phosphate within MV. With calcium stress, *Mgp* is upregulated to meet demand but if calcium stress persists, levels decrease and MV become mineralization-competent. MGP also aids in maintaining VSMC phenotype preventing a switch to that of an osteogenic potential (558,559). It is found in uncalcified vessels but its expression is downregulated at the onset of a fatty streak with some suggesting that this leaves the vessel wall vulnerable to unopposed BMP-2 action and therefore stimulation of VSMC to an osteogenic phenotype (473). A further interesting point is the "mineralization by inhibitor exclusion" concept (560,561). MGP may be necessary for inhibiting mineralization within elastin and collagen fibrils, an area where other inhibitors like Fetuin-A cannot access because of their size. Local VSMC must provide this calcification inhibitor as selective expression in the liver of knockout mice does not rescue the phenotype from vascular calcification (562). The loss of functional MGP is apparent in warfarin therapy where inhibition of the vitamin K-dependent formation of gla-residues results in an impotent inhibitor that is unable to bind calcium and phosphate ions, and vascular calcification ensues (563,564). This is also observed in cases of reduced levels of vitamin K and may be the case in aging as evidenced in rats (565), where circulating levels of uncarboxylated MGP are increased (566).

Transgenic mice lacking MGP die 1-3 months after birth due to rupture of large elastic arteries and also display endochondral ossification (558,559). Keutel syndrome is the corresponding process in humans. Individuals have abnormal cartilage calcification and short distal phalangeal lengths (brachytelephalangism) with life expectancy dependent on the severity of obstructive airway disease caused by extensive calcification of the tracheobronchial tree. Recent reports suggest neurological (encephalomalacia secondary to brain calcification), ophthalmologic (optic atrophy) and dermatological (mid-dermal elastolysis) involvement. Therefore, unlike the mouse model, deficiency of MGP in humans does not lead to widespread vascular calcification, instead the weight of its effect seems largely related to its ability to inhibit BMP-2 formation. This may be because of the recently identified calcification inhibitor Gla-rich protein which may rescue the vessel from calcification (567).

1.8.4.6.2. Pyrophosphate.

Inorganic pyrophosphate (PPi) is a potent inhibitor of calcium crystal deposition through its ability to adsorb to the surface of apatite crystals in phosphate positions and thereby prevent extension, to induce the expression of the mineralization inhibitor osteopontin and to inhibit alkaline phosphatase activity (529,568-570). It is the result of adenosine triphosphate (ATP) hydrolysis by the enzyme ectonucleotide pyrophosphatase/phosphodiesterase (ENPP).

ENPP enzyme is one of four major groups of ecto-nucleotidases of which the alkaline phosphatases are another both showing an alkaline pH optimum. There are seven known ENPPs with ENPP1-3 found in every cell type that has been analysed, and these possess the ability to degrade nucleotides hydrolysing ATP to AMP and PPi, although ENPP1 has the highest activity in this regard (571). Only ENPP1 is found in MV (572,573) and is implicated in the maintenance of VSMC phenotype, production of sRAGE (soluble endogenous

Suppressor of the Receptor for Advanced Glycation End-products), and the maintenance of serum phosphate and calcium through fibroblast growth factor-23 (574,575). ENPP1 is a transmembrane enzyme with a number of substrates including UDP-glucose, however it is its ability to hydrolyse the adenosine nucleosides that make it important in mineralization. ENPP1 is able to hydrolyze ATP, ADP and PPi as indicated in table 1 (493). ATP is the most efficiently hydrolysed compared with the other substrates. The extent to which the hydrolysis of PPi to Pi occurs is unknown. (571,576-579).

Table 1.8.1 ENPP1 hydrolysis of nucleotides important in mineralization. ATP adenosine triphosphate, PPi pyrophosphate, AMP adenosine monophosphate, Pi inorganic phosphate, ADP adenosine diphosphate.

ATP → PPi + AMP
ATP → Pi + ADP
ADP → Pi + AMP
PPi → 2xPi

ENPP1 prevents vascular calcification beyond its role in the formation of PPi. A recent discovery in mice implicates it in the regulation of *Klotho* expression under conditions of phosphate overload (580). Human VSMC also generate extracellular PPi via ENPP1 to abrogate vascular calcification. The importance of this inhibitor is highlighted in gene mutations of ENNP1 which lead to generalized arterial calcification of infancy with approximately 85% of infants dying by the age of six months from severe hypertension and cardiovascular complications of vascular calcification (581-583).

In the vasculature we see mechanisms providing extracellular and intra-vesicular PPi: ENPP1 and the membrane transporter ankylosis protein which allows intracellular PPi to be

transported out of the cell, working against the enzymes forming Pi: alkaline phosphatase, ENPP1 and 3 in certain situations, as well as intracellular phosphoethanolamine/phosphocholine phosphatase with the type III sodium-depending Pi co-transporters allowing Pi passage out of the cell, to prevent vascular calcification(574). Indeed, it is the relative expression of ENPP1 to alkaline phosphatases and the resulting balance of PPi and Pi that predetermines the final outcome of mineralization or mineralization inhibition (578,584). Therefore we see in bone formation that PPi is simultaneously used as a source of Pi in the control of terminal differentiation events of growth plate chondrocytes and necessary for mineralization to be seeded in MV, but in the absence of PPi hydrolysis by alkaline phosphatases, used to control the extent of mineralization (585). We also see PPi used as a Pi source in disease. In the heart, cardiac fibroblasts, like VSMC, have the potential to undergo phenotypic transformation into an osteogenic cell with the subsequent formation of a calcifying matrix. In this setting, ENPP1-generated PPi is hydrolysed to Pi enabling mineralization (586,587).

1.8.4.6.3. Fetuin-A.

At normal physiological concentrations of calcium and phosphate, serum is supersaturated and mineral precipitation would occur in the absence of systemic calcification inhibitors. Fetuin-A, a member of the Type 3 cystatins of the cystatin superfamily of protease inhibitors which comprise plasma proteins largely secreted by the liver, is one such systemic inhibitor (588,589). It is the observation that Fetuin-A had a high affinity for bone and that the addition of calcium and phosphate to sera removed measurable Fetuin-A that lead to the idea that this protein played a role in ectopic calcification (590-592).

Fetuin-A acts as a chaperone for calcium and phosphate. It does not inhibit the formation of mineral nuclei but prevents growth and aggregation of nuclei and therefore prevents

precipitation. Fetuin-A is first associated with posner clusters ($Ca_9(PO4)_6$) together called calciprotein monomers, which increase in size through aggregation forming the primary calciprotein particle which is a spherical nanoparticle containing amorphous calcium phosphate. Here Fetuin-A forms an outer layer similar to that of apolipoproteins shielding their lipid content. The secondary or mature calciprotein particle is a further enlarged needle-like structure with mineral crystalline particles. These particles can be cleared from serum by scavenger receptors in the reticuloendothelial system (468,593,594).

Naked calcium phosphate crystals are cytotoxic, Fetuin-A protects the vascular cell membrane from crystal induced damage and also protects the cell from apoptosis (475,505). Fetuin-A bound to the surface of these crystals confers some benefit but the soluble protein offers additional protection (505). Fetuin-A also prevents mineralization of matrix vesicles. VSMC take up extracellular Fetuin-A and package this as well as MGP into exosomal matrix vesicles. Under normal conditions these matrix vesicles do not calcify, however under mineralizing conditions such as increased extracellular calcium, matrix vesicles become mineralizing competent through the loss of their inhibitor proteins (507).

Apart from its crucial work in mineral chaperoning, Fetuin-A offers protection from ectopic mineralization by an anti-inflammatory effect (595,596) and through interference of TGF-β signalling. Interestingly, it possesses TGF-β receptor type II homology and at this site is able to bind TGF-β1 and TGF-β2 although it binds to BM2, BMP 4 and BMP 6 with greater affinity thereby reducing vascular calcification (597).

Vascular calcification occurs through a complicated interaction of proteins and cells through a variety of mechanisms exacerbated by age and underlying disease states which promote mineralization. Unequivocally associated with morbidity and mortality, unravelling its complexities will guide methods to attenuate its presence.

1.9. Adiponectin.

Although the idea of a signalling peptide originating from adipose tissue was proposed in 1953 (598) it was 40 years later before the first adipokine, leptin, was discovered (599). Since then a number of adipokines, including tumour necrosis factor-α, interleukin-6, plasminogen activator inhibitor-1, adiponectin and resistin have been discovered, establishing adipose tissue as an endocrine organ. Although many of the adipocytokines are pro-inflammatory and impair insulin sensitivity, adiponectin has anti-inflammatory and insulin-sensitizing effects.

Adiponectin was first identified in 1995 (600) simultaneously in both humans and mice. Unlike the discovery of leptin, the explosion in subsequent research did not occur until later that decade when the association between adiponectin and aspects of the metabolic syndrome were observed. We now know that adiponectin has a plethora of actions playing important roles in cellular energy pathways as well as displaying both anti-inflammatory and anti-apoptotic features. It is one mechanism by which adipose tissue exerts direct control over vascular function.

With the emerging realization that adipose tissue was not only the body's energy reservoir but an endocrine organ implicated in whole body energy metabolism, four independent groups using different methods discovered adiponectin. Lodish's group published their findings in 1995 naming the protein Adipocyte complement-related protein of 30 kDa (Acrp30) after identifying the protein by subtraction hybridization techniques with the generation of a cDNA library (600). The following year three further groups identified the amino acid sequence both in mouse and human samples (601-603).

Adiponectin is made up of an amino-terminal signal sequence, a short non-collagenous peptide, 22 collagen repeats and a carboxyl-terminal globular head which has significant homology with type VIII and X collagens. Although it is secreted predominantly by

adipocytes (both white adipose tissue and brown adipose tissue), adiponectin is made in many other tissues including the myocardium. It is synthesized as a single polypeptide (monomer) but undergoes multimerization within the endoplasmic reticulum to be secreted into the circulation as trimers (low molecular weight form – LMW), hexamers (medium molecular weight form - MMW) and high molecular weight (HMW) forms of 18-mers and larger in humans. These multimers appear to have cell specific interactions and, therefore, actions.

1.9.1. Multimerization.

Insulin resistance, diabetes mellitus and the metabolic syndrome (604-608), family history of diabetes mellitus (609,610), obesity (605,609), coronary heart disease (604,611,612) and multivessel atherosclerosis (613), hypertension (614), stroke, non-alcoholic fatty liver disease and steatohepatitis are all associated with reduced levels of adiponectin, as is male gender (604,607,615,616). Interestingly, this reduction is seen predominantly in the HMW fraction with the ratio of HMW to total adiponectin as opposed to total adiponectin levels, showing a strong negative correlation with the above disease states (601,607,617-619). When considering body fat distribution, intra-abdominal fat stores have the highest negative correlation with plasma adiponectin, compared with subcutaneous stores (615). Increasing serum adiponectin levels either by weight loss (604,620), by adiponectin therapy in animal models of diabetes (616,621), or pharmacologically (622) appears to reduce the risk for obesity-related complications. Considering how adipocytes construct high molecular weight adiponectin, therefore, becomes important when considering treatment options to increase the high order polymer to ameliorate disease.

Construction of adiponectin multimers occurs within the endoplasmic reticulum. The HSP70 (Heat Shock protein of 70 kDa) molecular chaperone BiP (Binding immunoglobulin protein) is important in the early folding and subsequent posttranslational modifications of the protein

monomer which itself is not found in the circulation as it is thought to be unstable (623). Trimeric adiponectin is the basic functional building block. Three monomers of adiponectin are joined by hydrophobic interactions within its globular head, stabilized by non-covalent interactions of the collagenous domains in a triple α-helix stalk. This structural form is remarkably similar to that of tumour necrosis factor-α, although the two proteins differ at the amino acid sequence level. Two homotrimers interact to form the hexameric MMW form. This occurs via a disulphide bond between cysteine residues (624) (Cys^{39} in humans) in the variable domain, an interaction that provides stability to intermediary oligomers during assembly of HMW species (625). Hydroxylation and glycosylation of lysine residues in the collagenous domain are also necessary for the HMW bouquet-like construction maintaining the secondary helical structure (616).

ERp44 (Endoplasmic reticulum resident protein 44) binds adiponectin at Cys^{39} facilitating further oxidative folding with Ero1-Lα (Endoplasmic oxireductin-1-like protein) and protein disulphide bond isomerase. Disulphide-bond A oxireductase-like protein (DsbA-L) also plays an important role in the formation of the HMW multimer aiding in the formation of intertrimeric disulphide bonds.

Once released from the endoplasmic reticulum into vesicles, cellular secretion appears to depend on the presence of GGA1 (Golgi-associated γ-adaptin ear homology domain Arf [ADP-ribosylation factor-interacting protein 1) as its absence within transport vesicles is associated with inhibition of adiponectin secretion. Upon secretion into the circulation, the different multimeric forms do not interconvert, highlighting the importance of the intra-cellular assembly machinery.

1.9.2. Receptors.

Adiponectin interacts with three receptors: AdipoR1, AdipoR2 and T-cadherin. AdipoR1 and AdipoR2 are similar to other G-protein coupled receptors containing 7-transmembrane domains, however, they have a reverse topology with an intracellular N-terminus and an extracellular C-terminus. T-cadherin is membrane-associated but lacks a transmembrane and an intracellular domain. Interestingly, although AdipoR1 and AdipoR2 are expressed in endothelial and vascular smooth muscle cells, T-cadherin is more abundantly expressed and absence of this adiponectin-binding protein mimics adiponectin deficiency in acute and chronic mouse models of cardiac injury (626). Adiponectin receptors also possess ceramidase activity, an enzyme that converts apoptosis-inducing ceramides into second messengers sphinganines and sphingosines, including sphingosine-1-phosphate, which is protective against insulin resistance, lending some insight into the complexity of adiponectin signalling and its plethora of downstream effects (627,628).

1.9.3. Cardiovascular effects.

Besides its insulin-sensitizing, anti-diabetic and muting effects on systemic inflammatory cytokines, adiponectin interferes with the initiation and propagation of crucial steps in the pathogenesis of atherosclerosis: endothelial dysfunction, endothelial expression of adhesion molecules and therefore monocyte adhesion, foam cell transformation of macrophages, smooth muscle cell proliferation and migration, matrix metalloproteinase activity, angiogenesis and endothelial and vascular smooth muscle cell apoptosis (613,629,630).

Endothelial dysfunction is crucial in atherosclerosis with decreases in relaxation increasing vascular tone leading to hypertension and altered flow dynamics promoting vessel damage. *In vitro*, adiponectin improves vessel relaxation by promoting the phosphorylation of nitric

oxide synthase (eNOS) and decreasing the reactive oxygen species superoxide and peroxynitrite (actually nitrotyrosine, the surrogate for peroxynitrite) (631).

Tumour necrosis factor-α induces endothelial expression of the monocyte adhesion molecules E-selectin, vascular cell adhesion protein-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1). Adiponectin attenuates this upregulation in a dose-dependent fashion encompassing physiological levels, subsequently decreasing monocyte adhesion to aortic endothelial cells reducing migration out of the circulation (611). Adiponectin also suppresses foam cell formation in macrophages by downregulating the expression of Class A scavenger receptors and therefore decreasing lipid accumulation and foam cell formation (632).

Vascular smooth muscle cell proliferation contributes to the growing atherosclerotic plaque, adiponectin is able to mitigate this both directly and indirectly. In a dose-dependent fashion, adiponectin attenuates PDGF-BB (platelet-derived growth factor composed of two B subunits) increases in vascular smooth muscle cell DNA synthesis and proliferation by directly binding to PDGF-BB extracellularly and by modulating the post-receptor extracellular signal-regulated kinases (ERK)-stimulation pathway (633). In an *in vivo* model of vessel injury, adiponectin inhibited proliferation and migration of vascular smooth muscle cells by inhibiting growth factor, and by preventing tumour necrosis factor-α-mediated increases in heparin-binding epidermal growth factor-like growth factor (HB-EGF) mRNA expression in endothelial cells (634). It may also prevent smooth muscle cell proliferation by increasing decorin synthesis (635).

Adiponectin increases tissue inhibitor of metalloproteinases-1 (TIMP-1) expression and secretion from mononuclear cells but not human aortic endothelial or smooth muscle cells and, by extension, likely inhibits the actions of locally occurring matrix metalloproteinases.

This action was interleukin-10-dependent as treatment with anti-interleukin-10 completely abrogated the adiponectin-induced TIMP-1 mRNA expression (636).

Angiogenesis in the atherosclerotic plaque is associated with macrophage infiltration, intraplaque haemorrhage and thin-cap lesions however, a causal relationship has yet to be defined (637). Vascular endothelial growth factor (VEGF) induces angiogenesis by promoting proliferation and growth of endothelial cells in arteries, veins and lymph vessels. In situations of low oxygen tension, which may be the case in the growing human atherosclerotic plaque, endothelial cells secrete VEGF and upregulate VEGF receptors (638). In monocytes VEGF stimulates the secretion of the pro-inflammatory cytokines tumour necrosis factor-α and interleukin 1β as well as growth factors inducing endothelial proliferation. Adiponectin is able to abrogate the effects of VEGF on human coronary artery endothelial cells. Predominantly mediated by cyclic adenosine 3', 5'-monophosphate/protein kinase A (cAMP/PKA) pathway, adiponectin reduced the production of reactive oxygen species and the increased extracellular signal-regulated kinases (ERK) and Protein kinase B (Akt) phosphorylation that induced endothelial cell migration (639).

Adiponectin also binds to subendothelial thrombogenic stimuli collagen I, III and IV abrogating platelet activation (640), and blocks calcification induced by vascular smooth muscle cells in response to the combined stimulation by tumour necrosis factor-α and phosphate (641). It also reduces endothelial cell apoptosis (642) which may explain why adiponectin is negatively correlated with the necrotic core of atherosclerotic plaques in patients with acute coronary syndrome (643).

In the heart, adiponectin is able to prevent cardiac hypertrophy induced by pressure overload (644) and abrogate the fibrosis and left ventricular dysfunction induced by angiotensinogen II (645). It is also able to reduce tumour necrosis factor-α production, apoptosis and infarct size

associated with an ischaemic-reperfusion injury which it mediates through COX2 (646), T-cadherin (626) and an upregulation of VEGF (630). Like its action in the endothelium, it is also able to reduce oxidative stress in cardiomyocytes (647).

Given the assertive role adiponectin plays against the pathogenesis of atherosclerosis, in theory, the adiponectin knockout mouse appears to be well suited as an *in vivo* model of a pro-atherogenic milieu. However, preclinical models may present unexpected and conflicting findings due to either a true response or differences in the experimental parameters, diet or other unknown factors (648). With these caveats in mind we utilized the adiponectin knockout mice for our investigation of the effect of calcium supplements on the vasculature.

2. THE AUCKLAND CALCIUM STUDY: 5-year post-trial follow-up.

2.1. Introduction

Calcium supplements are advocated for the treatment and prevention of osteoporotic fractures. However, studies of their effects on bone mineral density (BMD) and fracture incidence have shown only small benefits (300,649). In the Auckland Calcium Study, a 5-year randomised, placebo-controlled trial of calcium supplements, we observed no effect of calcium on total, forearm or vertebral fracture incidence but an increase in hip fractures with calcium. In women allocated to calcium, BMD increased at the lumbar spine, and there was decreased BMD loss at the hip and total body (650). In a secondary analysis with pre-specified cardiovascular endpoints (288), there was a significant increase in the rate of the composite cardiovascular endpoint of myocardial infarction (MI), stroke or sudden death in women allocated to calcium. These unexpected findings prompted a meta-analysis of all existing trials of calcium monotherapy revealing a 27-31% increase in MI and a non-significant increase in the risk for stroke (12-20%) with calcium (289).

Therefore, we continued follow-up of the Auckland Calcium study participants post-trial to identify whether the effects of calcium supplements observed in the trial persisted after supplement discontinuation. Specifically, we investigated post-trial fracture incidence and whether the increased risk for hip fractures persisted, whether there were lasting effects on BMD, and whether the harmful effects of calcium supplements on cardiovascular risk continued after their cessation.

2.2. Materials and methods

2.2.1. Participants

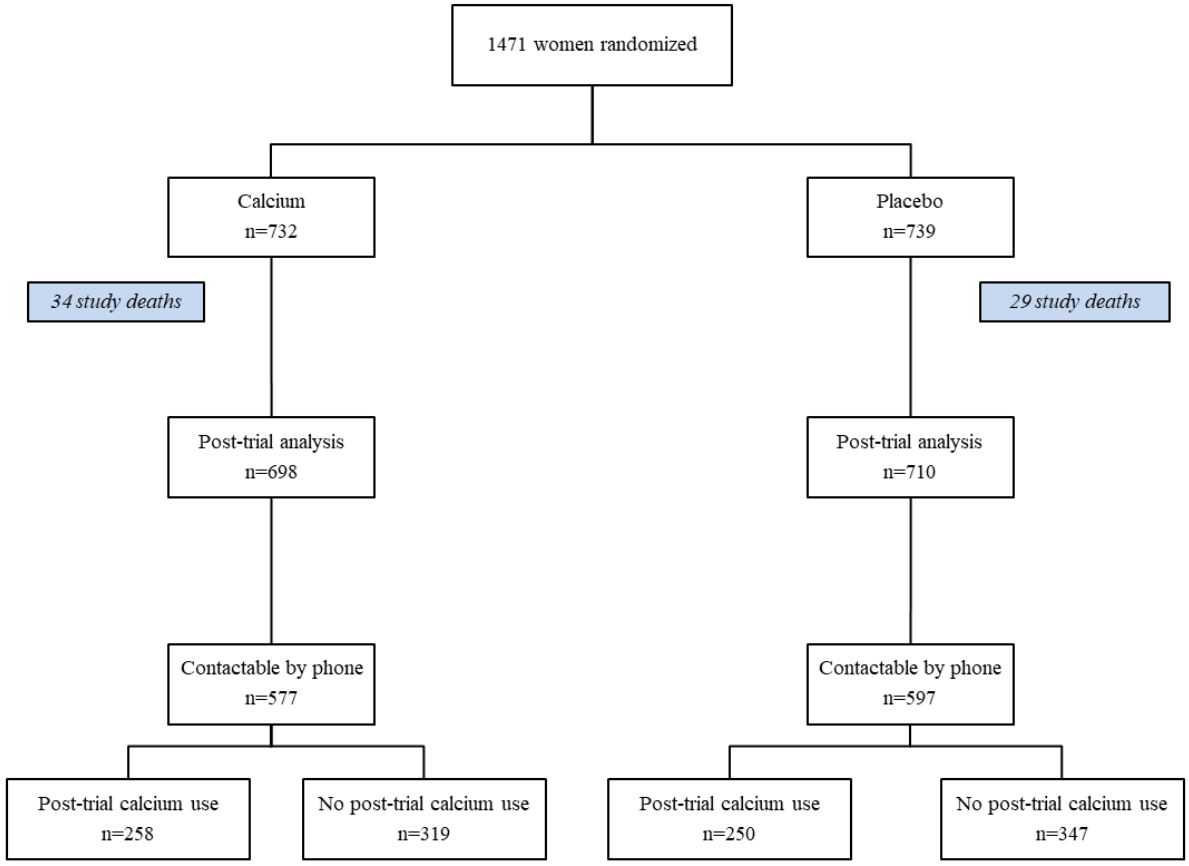
1471 healthy, postmenopausal women participated in a 5-year, randomised, double-blind, placebo-controlled trial of calcium supplements. The primary endpoint of the study was time

to first clinical fracture. Individual fracture sites, bone mineral density (BMD), and cardiovascular endpoints were pre-specified as secondary endpoints. The study design and results have being described previously (650). Briefly, women were included if they were aged >55 years and had normal lumbar spine BMD for their age, and were excluded if they were receiving treatment for osteoporosis or taking calcium supplements, had any other major ongoing disease, or had serum 25-hydroxyvitamin D levels <25 nmol/L. The original trial started in 1998 and was completed in 2005. Participants were randomised to either 1 g of elemental calcium daily as the citrate (Citracal; Mission Pharmacal, San Antonio, TX) or identical placebo. Between mid-2008 and mid-2009, all surviving study participants were contacted by telephone, and in early 2010, searches of the national hospital admissions and national mortality datasets were undertaken for relevant hospital admissions and for deaths. 63 women died during years 1-5 of the study, leaving 1408 women as the starting cohort for this post-trial follow-up study. Of these, 194 died during the post-trial period and 1174 (83%) were able to be contacted by phone. 29 women did not have a relevant hospital admission and could not be contacted (Figure 1). We included data from all 1408 women in the post-trial analyses, and data from all 1471 women in analyses of the entire 10-year follow-up. Both the original and post-trial studies were approved by the Northern X Regional Ethics Committee.

2.2.2. Measurement, cardiovascular events, and fractures

At trial entry, medical history was obtained by questionnaire; weight was measured using electronic scales, and height using a Harpenden stadiometer. Other laboratory and dietary measures were obtained as previously described (650). BMD of the lumbar spine, femoral neck, and total body was measured at baseline, 2.5 years, and 5 years using a Lunar Expert dual-energy x-ray absorptiometer (DXA), software version 1.7 (GE Lunar, Madison WI).

Figure 2.1 Flow of participants



Participants were seen every six months, and adverse events were recorded, although specific events or symptoms were not inquired about. At the end of the study, a search of the national database was undertaken for unreported myocardial infarctions (MI) or strokes, and both these unreported events and all self-reported cardiovascular events were independently adjudicated as previously described (288). During the initial 5-year randomised trial, participants were asked at each 6-month visit about fractures, and relevant radiographs or reports were reviewed.

Osteoporotic fractures were defined as all fractures except those of the head, hands, feet and ankles, and those resulting from major trauma.

In the post-trial follow-up, details of any fractures, cardiovascular events, other medical events and medications since trial completion were recorded at the single phone interview, and hospital admissions related to cardiovascular and fracture events (ICD 9 discharges codes 410-414, 430-438, 798, and 800-829) were obtained from the searches of the national hospital admissions database. For these analyses, we have only considered the incident event for each outcome. The date of the first event was considered the date of the first hospital admission for that event, or if there were no hospital admissions, the date of the first self-reported event. The final date of follow-up for the post-trial study was the date of phone contact, and where there was no phone contact, the last date out of the date of death, the date of the last hospital admission, or the date of completion of the original study.

2.2.3. Bone mineral density sub-study

At the end of the original trial, all participants were given general management advice for osteoporosis: those with normal BMD were given general advice; those with osteopenia were recommended to take calcium supplements, and those with osteoporosis to take bisphosphonate treatment (Table 1). To determine whether the benefits observed in the original trial persisted after calcium was stopped, we measured BMD at 10 years in 193 women who remained on trial medication to 5 years in the original trial and who had not taken bone-active medication, including calcium supplements, since trial completion. Based on the original trial, we calculated that a sample size of 200 subjects would be adequate to detect between-group differences in bone density of at least 0.5%. Due to spinal and hip surgery, a number of patients had to be excluded from the final analyses reducing the number to 193. The slight reduction in sample size does not affect the minimum detectable difference.

Table 2.1 Bone mineral density status at study completion.

Total body	**Normal**	**Osteopenic**	**Osteoporotic**	**Total**
Calcium	39	24	14	77
Placebo	32	47	21	100
Total femur				
Calcium	38	25	11	74
Placebo	32	47	18	97
Lumbar spine				
Calcium	39	24	14	77
Placebo	30	48	22	100
Overall				
Calcium	39	25	17	81
Placebo	33	50	29	112
Total	72	75	46	193

For each BMD site, we restricted analyses to women who had a BMD measurement at all four time points (baseline, 2.5 years, 5 years and 10 years). DXA availability meant that a small proportion of DXA scans at 5 years and all scans at 10 years were performed on a Prodigy DXA (GE, Madison, WI). Prodigy results were recalibrated to match data obtained from the Expert platform used for the majority of in-study measurements. Site-specific, ordinary least squares regression equations were fitted to Expert (dependent variable) and Prodigy (independent variable) BMD data for 64 participants from an unrelated study. Inspection of residuals and goodness-of-fit statistics verified the adequacy of these fits. Using these equations, Prodigy results were converted to align with values obtained using the Expert DXA.

Table 2.2 Baseline characteristics of participants who entered the post-trial period by original treatment allocation.

Characteristics	**Calcium n=698**	**Placebo n=710**
Age (years)	74.1 (4.2)	74.1 (4.2)
Weight (kg)	67.0 (11.4)	67.2 (11.4)
Body mass index (kg/m^2)	26.6 (4.3)	26.5 (4.2)
Glomerular filtration rate (ml/min/1.73m^2)	61 (11)	61 (10)
Dietary calcium (mg/day)[a]	865 (392)	854 (382)
Total calcium (mmol/l)	2.32 (0.07)	2.31 (0.07)
Serum 25-hydroxyvitamin D (µg/L)	22 (7)	22 (7)
Glucose (mmol/l)	5.1 (0.7)	5.1 (0.7)
Cholesterol (mmol/L)[b]		
Total	6.6 (1.2)	6.5 (1.0)
High density lipoprotein cholesterol (mmol/l)	1.7 (0.5)	1.6 (0.4)
Low density lipoprotein cholesterol (mmol/l)	4.2 (1.2)	4.2 (1.0)
Triglycerides (mmol/l)	1.5 (0.8)	1.7 (1.0)
Bone mineral density (g/cm^2)		
Lumbar spine	1.06 (0.18)	1.05 (0.18)
Lumbar spine T score	-1.0 (1.5)	-1.1 (1.5)
Total hip	0.86 (0.14)	0.86 (0.13)
Total hip T score	-1.2 (1.1)	-1.2 (1.1)
Total body	1.04 (0.09)	1.03 (0.09)
Physical activity (METS)	34 (4.7)	33 (4.2)
Systolic blood pressure (mmHg)	137 (22)	136 (22)
Diastolic blood pressure (mmHg)	71 (11)	70 (10)
Smoking status		
Current (%)	3	2.5
Former (%)	40	37
Medical history		
Previous hypertension (%)	30	28
Previous ischaemic heart disease (%)	8	7
Previous stroke\transient ischaemic attack (%)	1	0
Dyslipidaemia (%)	10	8
Diabetes (%)	2.3	2.7
Previous fracture (%)	28	29

[a] By validated food frequency

[b] Lipids were measured in fasting blood samples in a subset of 237 postmenopausal women. 118 were assigned to calcium supplementation, and 119 to placebo

2.2.4. Statistics

Baseline characteristics were compared between the groups using Student's *t* test for continuous variables and the Chi-square test for categorical variables. Kaplan-Meier curves were used to compare the proportion of women in each group experiencing an incident event over time, and Cox proportional hazard models to compare survival curves with adjustment for baseline differences where appropriate. The assumption of proportional hazards was explored by performing a test for proportionality of the interaction between variables included in the model and the logarithm of time. For BMD data, we pre-specified a comparison of the percentage change from baseline at 10 years with Student's *t*-test. Statistical analyses were performed using the SAS software package version 9.2 and all analyses were based on intention to treat unless otherwise stated. $p < 0.05$ was considered significant and all tests were two-tailed.

2.3. Results

2.3.1. Participants

The mean duration of follow up in the post-trial period was 4.8 years (SD 2.0 years), and the mean duration of follow-up from baseline was 9.1 years (SD 2.0 years). Figure 2.1 shows the flow of participants through the trial and post-trial periods. The characteristics at entry to the original trial of the 1408 women who continued in post-trial observation are shown in Table 2.2. There were no statistically significant differences between the calcium and placebo groups. Women who did not enter the post-trial observation because they died during the original study were older, weighed less, had worse renal function, and were more likely to smoke than women who entered the post-trial observation (Table 2.3). We obtained information on medication use in the post-trial period in 1174 women. Of these, 43% used calcium supplements (51% of these had a trial allocation to calcium and 49% to placebo), and

33% used bisphosphonates (trial allocation to calcium: 50%; trial allocation to placebo: 50%). No women reported using hormone replacement therapy.

Table 2.3 Baseline characteristics of participants who did and did not enter the post-trial period. Values are mean (standard deviation).

Characteristics	**Post-trial participants *n* = 1408**	**Non-post-trial participants *n* = 63**
Age (years)	74.1 (4.2)	77.1 (4.6)
Weight (kg)	67.1 (11.2)	63.7 (11.6)
Body mass index (kg/m^2)	26.5 (4.2)	25.4 (4.5)
Glomerular filtration rate (ml/min/1.73m^2)	61 (10)	57 (14)
Dietary calcium (mg/day)[a]	860 (387)	808 (356)
Total calcium (mmol/l)	2.32 (0.09)	2.33 (0.10)
Serum 25-hydroxyvitamin D (µg/L)	22 (7)	20 (7)
Glucose (mmol/l)	5.1 (0.7)	5.3 (0.9)
Cholesterol (mmol/L)[b]		
Total	6.6 (1.1)	7.1 (1.8)
High density lipoprotein cholesterol (mmol/l)	1.6 (0.4)	1.6 (0.3)
Low density lipoprotein cholesterol (mmol/l)	4.2 (1.1)	4.8 (1.5)
Triglycerides (mmol/l)	1.6 (0.9)	1.7 (1.0)
Bone mineral density (g/cm^2)		
Lumbar spine	1.06 (0.18)	1.07 (0.19)
Lumbar spine T score	-1.0 (1.5)	-0.95 (1.6)
Total hip	0.86 (0.13)	0.81 (0.12)
Total hip T score	-1.2 (1.1)	-1.6 (1.0)
Total body	1.03 (0.09)	1.02 (0.09)
Systolic blood pressure (mmHg)	141 (23)	146 (24)
Diastolic blood pressure (mmHg)	72 (11)	74 (12)
Smoking status		
Current (%)	3	8
Former (%)	39	43
Medical history		
Previous hypertension (%)	29	33
Previous ischaemic heart disease (%)	8	11
Previous stroke/transient ischaemic attack (%)	1	3
Dyslipidaemia (%)	9	5
Diabetes (%)	3	6
Previous fracture (%)	28	33

[a] By validated food frequency

[b] Lipids were measured in fasting blood samples in a subset of 237 postmenopausal women. 118 were assigned to calcium supplementation, and 119 to placebo

2.3.2. Fractures

There was no statistically significant difference in the incidence of total fractures between the groups for the entire follow-up period (hazard ratio: 0.90, 95% confidence interval: 0.75-1.07, $p = 0.23$, Table 2.4). Figure 2.2 shows the time course of fracture subtypes by original treatment allocation during the entire follow-up period. There was a significant reduction in forearm and vertebral fractures with calcium, while the increased risk in hip fracture with calcium seen in the original trial was no longer statistically significant.

When analyses were restricted to the post-trial period, there was a significant reduction in vertebral and osteoporotic fractures in those originally allocated to calcium, but no effect for hip or forearm fractures (Table 2.4). When these analyses were further restricted to events obtained from the national hospital admission dataset, the results changed minimally (Table 2.5). We constructed a series of Cox proportional hazard models of the post-trial period for time to incident fracture firstly restricted to women who did not take calcium in the post-trial period, secondly for only those women who took a calcium supplement in the post-trial period and thirdly excluding the 387 women who used a bisphosphonate (193 study allocation to calcium, 194 study allocation to placebo; Table 2.6). In these analyses, there were no between group differences in fracture outcomes.

A final stratified analysis according to study treatment allocation and post-trial calcium use identified women who were allocated to calcium during the study and who took post-trial calcium to be at increased risk for hip fractures. Women who were allocated to the placebo arm in the trial and who took post-trial calcium were also at an increased risk for osteoporotic fractures.

Figure 2.2 Cumulative incidence of fractures for the entire follow-up period. Results are unadjusted. Participants at risk at each time point are represented below the graph. CI: confidence interval.

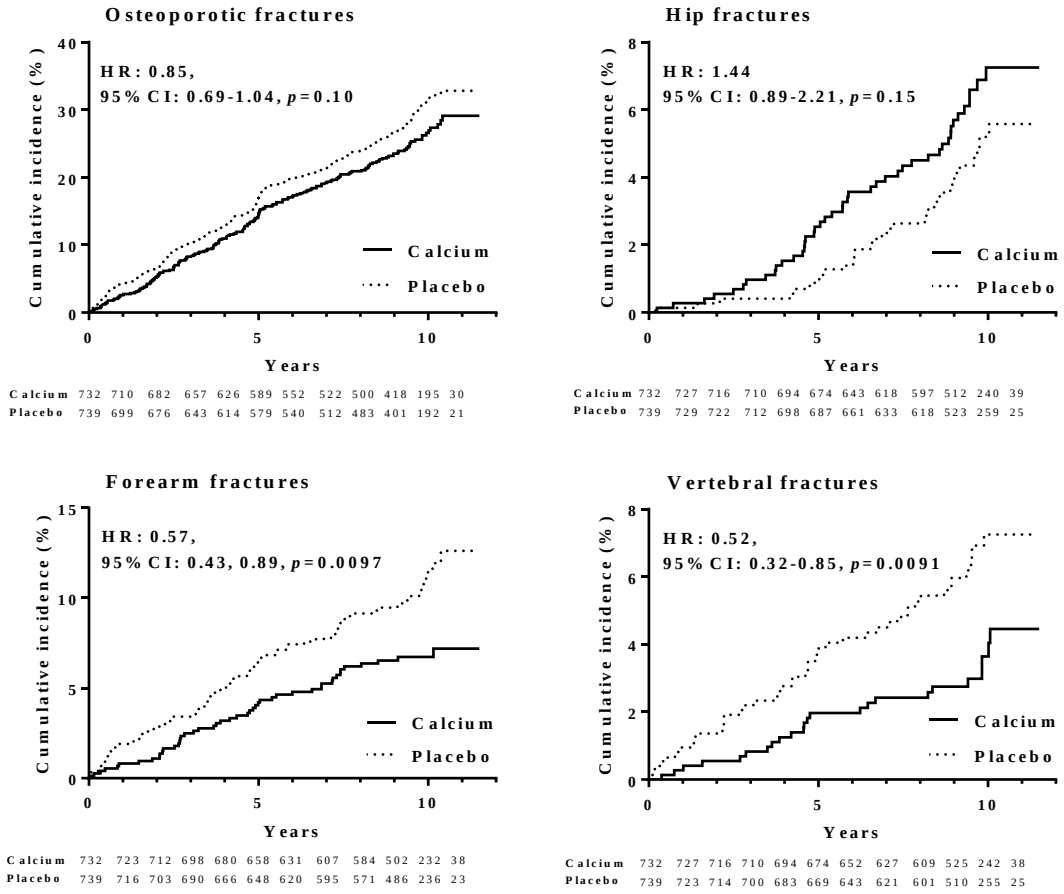


Table 2.4 Incident fracture and vascular events over the post-trial period and entire follow-up period. Results are unadjusted.

	Post-trial				Entire follow-up			
Event	**Calcium (*n* = 698)**	**Placebo (*n* = 710)**	**Hazard ratio (95% CI)**	***p*-value**	**Calcium (*n* = 732)**	**Placebo (*n* = 739)**	**Hazard ratio (95% CI)**	***p*-value**
Any fracture	121	139	0.86 (0.68, 1.10)	0.24	225	246	0.90 (0.75-1.07)	0.23
Osteoporotic fracture	91	119	0.76 (0.58, 1.00)	**0.047**	179	207	0.85 (0.69-1.04)	0.10
Forearm fracture	21	34	0.61 (0.36, 1.07)	0.086	47	75	0.62 (0.43-0.89)	**0.0097**
Vertebral fracture	10	22	0.46 (0.22, 0.97)	**0.041**	24	46	0.52 (0.32-0.85)	**0.0091**
Hip fracture	29	27	1.09 (0.64, 1.84)	0.75	44	32	1.40 (0.89-2.21)	0.15
Myocardial infarction	43	52	0.83 (0.56, 1.25)	0.38	70	68	1.04 (0.74-1.45)	0.83
Stroke	50	59	0.86 (0.59, 1.26)	0.44	80	78	1.04 (0.76-1.42)	0.81
Transient ischaemic attack	39	35	0.82 (0.51, 1.33)	*	58	53	1.12 (0.77, 1.62)	0.57
			2.89 (0.58, 14.3)	*				
Death	104	90	1.16 (0.87, 1.54)	0.31	138	119	1.16 (0.91-1.48)	0.24

CI: confidence interval

* Proportion assumption violated. Inspection of lifetest plots revealed plot crossover at 10 years and therefore a single mode with two hazard ratios was generated, the first from 0-10 years and the second 10 years <.

Table 2.5 Incident fracture and vascular events over the post-trial period and entire follow-up restricted to data obtained from the national database of hospital admissions. Results are unadjusted.

	Post-trial				Entire follow-up			
Event	**Calcium (*n* = 698)**	**Placebo (*n* = 710)**	**Hazard ratio (95% CI)**	***p*-value**	**Calcium (*n* = 732)**	**Placebo (*n* = 739)**	**Hazard ratio (95% CI)**	***p*-value**
Any fracture	69	91	0.75 (0.55, 1.03)	0.077	188	213	0.87 (0.71, 1.06)	0.15
Osteoporotic fracture	57	81	0.70 (0.50, 0.99)	**0.041**	150	179	0.82 (0.66, 1.02)	0.078
Forearm fracture	9	18	0.50 (0.23, 1.12)	0.092	36	62	0.57 (0.38, 0.86)	**0.0079**
Vertebral fracture	3	12	0.25 (0.07, 0.89)	**0.033**	17	36	0.47 (0.26, 0.84)	**0.010**
Hip fracture	26	24	1.10 (0.63, 1.91)	0.75	41	29	1.44 (0.90, 2.32)	0.13
Myocardial infarction	39	44	0.90 (0.58, 1.38)	0.61	66	62	1.07 (0.76, 1.52)	0.69
Stroke	39	43	0.92 (0.60, 1.42)	0.71	69	64	1.09 (0.78, 1.53)	0.62
Transient ischaemic attack	19	9	2.16 (0.98, 4.77)	0.06	41	28	1.49 (0.92, 2.40)	0.11

CI: confidence interval

Table 2.6 Hazard ratio for fracture and cardiovascular outcomes in the post-trial period by use of calcium or bisphosphonate. Results are unadjusted.

Restricted to women who did not take calcium post-trial (*n* = 666)

Event	Calcium (*n* = 319)	Placebo (*n* = 347)	Hazard Ratio (95% CI)	*p*-value
Osteoporotic fracture	27	45	0.64 (0.40, 1.04)	0.07
Forearm fracture	9	19	0.51 (0.23, 1.14)	0.10
Vertebral fracture	3	8	0.41 (0.11-1.55)	0.19
Hip fracture	4	6	0.72 (0.20, 2.57)	0.62
MI	14	21	0.73 (0.37, 1.43)	0.36
Stroke	16	22	0.80 (0.42, 1.52)	0.49
TIA	15	20	0.82 (0.42, 1.61)	0.57

Restricted to women who took calcium post-trial (*n* = 508)

Event	Calcium (*n* = 258)	Placebo (*n* = 250)	Hazard Ratio (95% CI)	*p*-value
Osteoporotic fracture	51	58	0.83 (0.57, 1.21)	0.33
Forearm fracture	11	15	0.71 (0.33, 1.55)	0.39
Vertebral fracture	6	11	0.53 (0.20, 1.43)	0.21
Hip fracture	19	12	1.55 (0.75, 3.20)	0.23
MI	11	14	0.76 (0.35, 1.68)	0.50
Stroke	12	16	0.73 (0.35, 1.55)	0.42
TIA	18	14	1.27 (0.63, 2.56)	0.50

Restricted to women who did not take a bisphosphonate post-trial (*n* = 787)

Event	Calcium (*n* = 384)	Placebo (*n* = 403)	Hazard Ratio (95% CI)	*p*-value
Osteoporotic fracture	29	45	0.67 (0.42, 1.07)	0.10
Forearm fracture	8	19	0.44 (0.19, 1.01)	0.052
Vertebral fracture	4	7	0.61 (0.18, 2.07)	0.43
Hip fracture	5	7	0.75 (0.24, 2.37)	0.63

CI: confidence interval

2.3.3. Bone mineral density

193 women had BMD measurements performed at a mean of 5 years following trial completion. For total femur, 171 women had data for all time points, whereas for lumbar spine and total body, 177 women had data for all time points. The characteristics of these women differed from the original cohort, because of the selection criteria. As a result, they were younger, had a higher body mass index and had higher baseline BMD measurements than the entire cohort, and the BMD results of this subgroup differ from those of the entire cohort in the original randomised controlled trial (Figure 2.3). At 10 years, there were no statistically significant differences in the percentage change from baseline between the groups at any BMD site (Figure 2.4).

2.3.4. Cardiovascular events

There were no statistically significant differences between the groups in the incidence of MI (hazard ratio: 1.04, 95% confidence interval: 0.74-1.45, $p = 0.83$), stroke (hazard ratio: 1.04, 95% confidence interval: 0.76-1.42, $p = 0.81$), transient ischaemic attack (hazard ratio: 1.12, 95% confidence interval: 0.77-1.62, $p = 0.57$) or death (hazard ratio: 1.16, 95% confidence interval: 0.91-1.48, $p = 0.24$) over the entire follow-up period (Figure 2.5). There were also no significant differences in these endpoints when analyses were restricted to the post-trial period (Table 2.4). When these analyses were further restricted to events obtained from the national hospital admission dataset, the results were little changed (Table 2.5).

Figure 2.3 Raw bone mineral density data of entire cohort and post-study participants. Post-study participants are those women who did not take calcium supplements of a bisphosphonate after completion of the original trial. *p*-values represent between-group differences at 10 years in post-study participants. Values are mean ± 95% confidence interval.

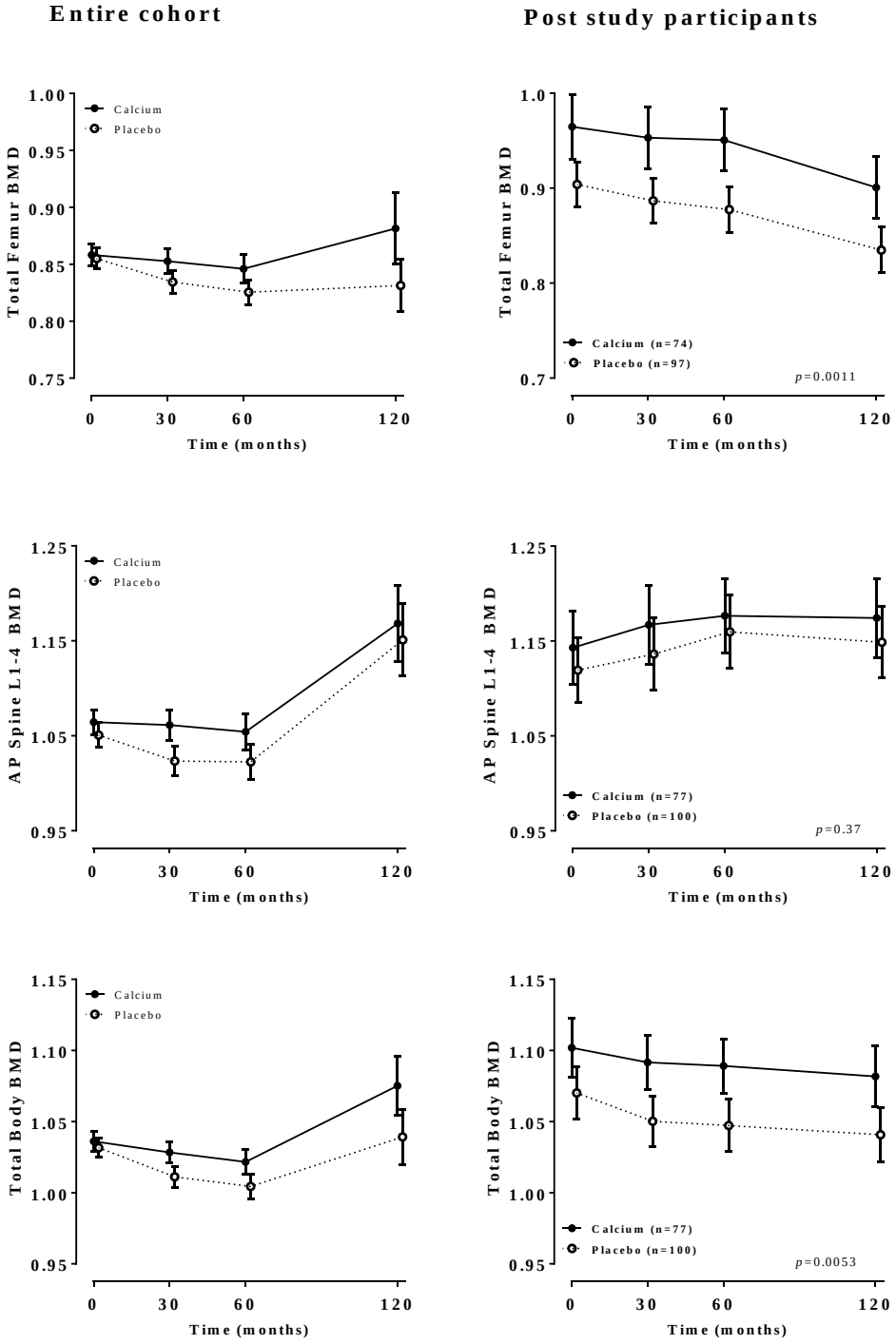


Figure 2.4 Percentage change in bone mineral density values from baseline in women who did not take calcium supplements or a bisphosphonate after completion of the original trial. *p*-values represent between-group differences in percentage change from baseline at 10 years. Values are mean (standard error of the mean).

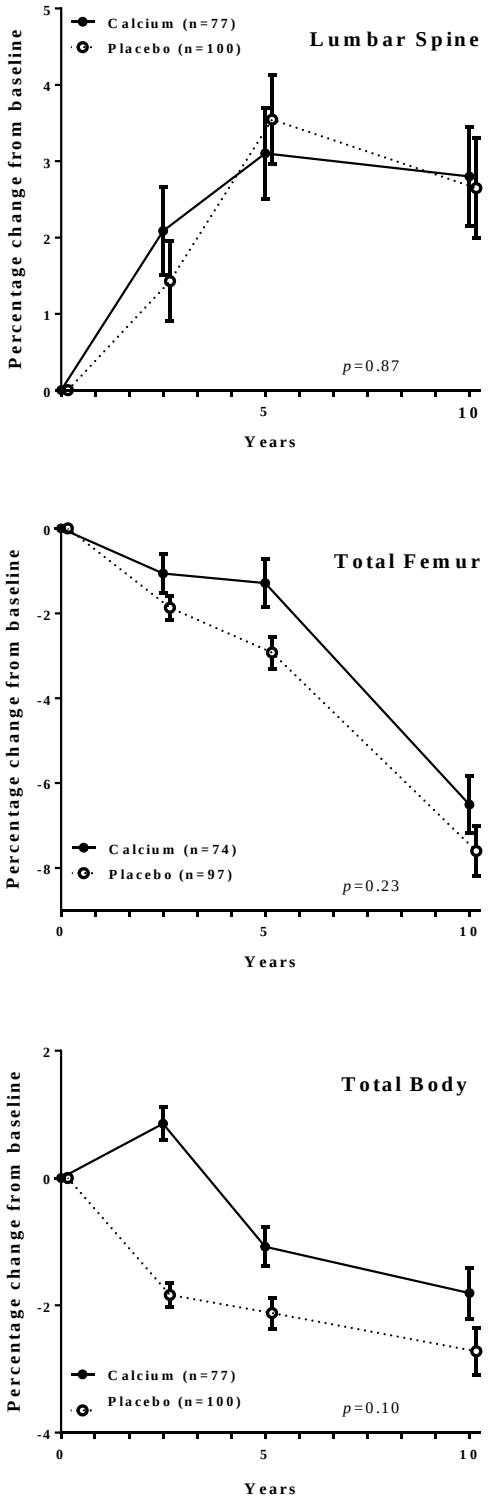
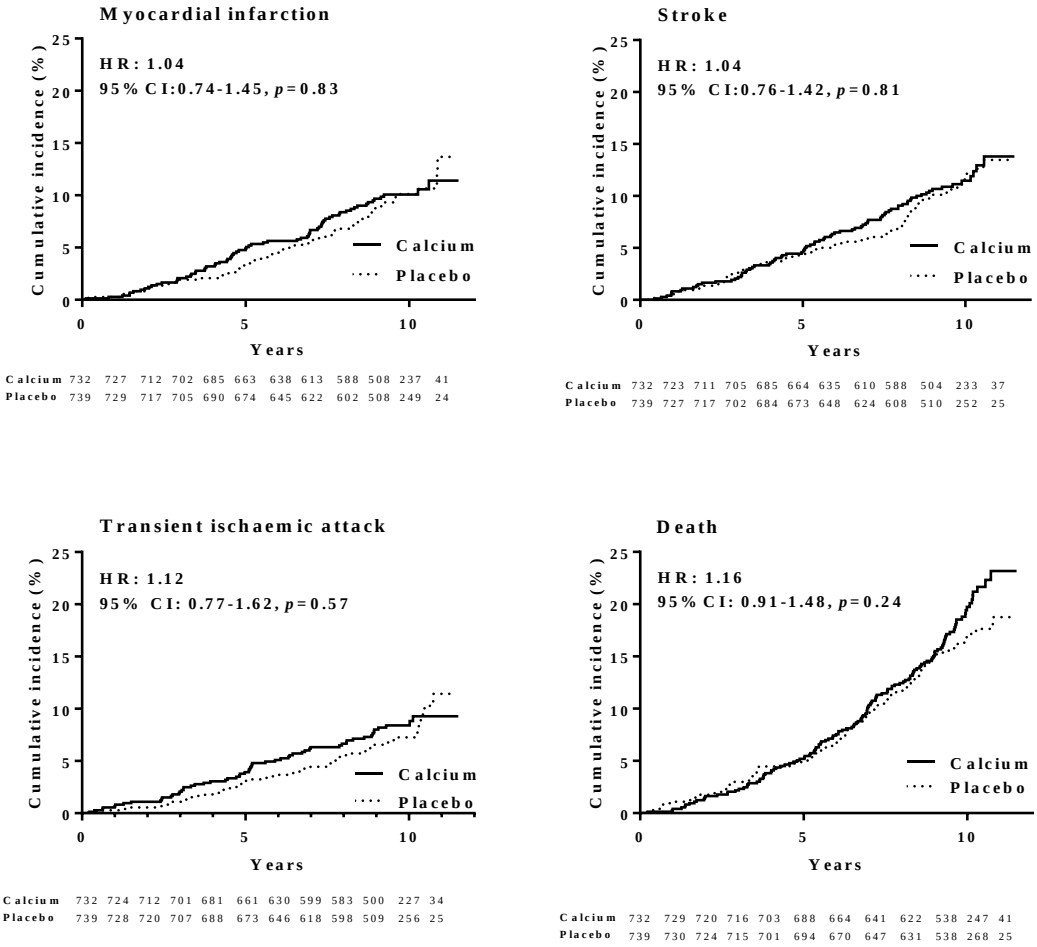


Figure 2.5 Cumulative incidence of cardiovascular events and mortality for the entire follow-up period. Results are unadjusted. Participants at risk at each time point are represented below the graph. CI: confidence interval.



We explored the potential effects of post-trial calcium use on cardiovascular events using a series of Cox proportional hazard models for time to incident cardiovascular event. Firstly, in 666 women known to have not used calcium in the post-trial period, original allocation to calcium was not associated with altered risk of MI (hazard ratio: 0.73, 95% confidence interval: 0.37-1.43), stroke (hazard ratio: 0.80, 85% confidence interval: 0.42-1.52) or TIA (hazard ratio: 0.82, 95% confidence interval: 0.42-1.61) in the post-trial period (Table 2.6). In an opposing analysis of the 508 women who took a calcium supplement in the post-trial period, original allocation to calcium was not associated with altered risk of MI (hazard ratio: 0.76, 95% confidence interval: 0.35-1.68), stroke (hazard ratio: 0.73, 95% confidence interval: 0.35-1.55) or TIA (hazard ratio: 1.27, 95% confidence interval: 0.63-2.56) in the post-trial period (Table 2.6).

Finally, in a stratified analysis according to trial allocation and post-trial calcium use, no group had an increased risk for MI, stroke or TIA compared with the referent group of trial allocation to placebo and no post-trial calcium use (Table 2.7). For all of these analyses, there were no significant between-group differences.

Table 2.7 Hazard ratios for fracture and cardiovascular outcomes in the post-trial period stratified according to trial allocation and post-trial calcium use. Results are unadjusted unless otherwise stated.

Grouped by treatment allocation and use of calcium supplements post-trial.

	Trial calcium, post-trial calcium use (*n* = 258)	Trial calcium, no post-trial calcium use (*n* = 319)	Trial placebo, post-trial calcium use (*n* = 250)	Trial placebo, no post-trial calcium use (*n* = 347)
Osteoporotic fracture	51	27	58	45
Forearm fracture	11	9	15	19
Vertebral fracture	6	3	11	8
Hip fracture	19	4	12	6
MI	11	14	14	21
Stroke	12	16	16	22
TIA	18	15	14	20
	Hazard Ratio (95% CI)[a]			
Osteoporotic fracture	1.46 (0.97, 2.18)	0.68 (0.42, 1.09)	1.89 (1.27, 2.78)[b]	1
Forearm fracture	0.73 (0.35, 1.53)	0.52 (0.23, 1.14)	1.06 (0.53, 2.09)	1
Vertebral fracture	0.85 (0.29, 2.46)	0.40 (0.11, 1.49)	1.59 (0.64, 4.00)	1
Hip fracture	3.58 (1.43, 9.00)[a]	0.72 (0.20, 2.57)	2.22 (0.83, 5.95)	1
MI	0.69 (0.33, 1.44)	0.73 (0.37, 1.43)	0.91 (0.46, 1.79)	1
Stroke	0.73 (0.36, 1.48)	0.80 (0.42, 1.52)	1.00 (0.53, 1.91)	1
TIA	1.23 (0.65, 2.33)	0.82 (0.42, 1.60)	0.97 (0.49, 1.92)	1

[a] Fracture outcomes adjusted for age and body mass index

[b] $p < 0.005$; CI = confidence interval

2.4. Discussion

In women randomly assigned to calcium supplements or placebo for 5 years with a further 5 years of post-trial follow-up, there was no effect of calcium on total or osteoporotic fractures, but significant reductions in forearm and vertebral fractures with calcium for the entire follow-up period. The adverse effect of calcium on hip fractures seen in the original study did not persist in the post-trial follow-up. However, in a stratified analysis, women who were originally allocated to calcium and continued to take a calcium supplement in the post-trial period had an increased risk of hip fracture remarkably similar to that of the original trial (hazard ratio, 3.58 vs 3.55 in the original trial) when compared with women who were originally allocated to placebo and did not take a calcium supplement in the post-trial period. The benefits on BMD from calcium observed in the original trial were no longer present at 10 years. The adverse cardiovascular effects observed in the original trial did not persist post-trial.

Calcium supplements reduce total fracture risk by a small amount. Although the findings of individual trials vary (650-653), a meta-analysis by Tang *et al.* (300) found a 10% risk reduction for total fracture with calcium monotherapy and a 13% risk reduction for total fracture with co-administered calcium and vitamin D. However, calcium monotherapy does not prevent hip fracture (305,654), and co-administered calcium and vitamin D prevents hip fractures in elderly institutionalized women but not community dwelling women (655). It is not certain whether any effects of calcium on fracture risk persist after supplements are discontinued. Bischoff-Ferrari *et al.* reported that calcium supplements reduced fracture risk during 4 years of a randomised, placebo-controlled trial but not over 6 years of post-trial follow-up, although total fracture numbers were small (652). While there were no significant effects on total fractures and hip fracture, we observed a reduction in forearm and vertebral

fractures over the entire 10-year follow-up. The cause of these latter findings is unclear. It may be a chance finding, or may represent a specific effect of calcium supplements. Until the findings are confirmed in other studies, they should be interpreted cautiously. In our original 5 year study, there was a significant increase in hip fracture with calcium, and similar but not statistically significant results were seen in two other trials of calcium monotherapy (305). There was no evidence of persisting increased hip fracture risk post-trial once calcium supplements had been discontinued in our current analyses. Overall, the results suggest that any effects of calcium supplements on fracture incidence do not outlast the period of supplement use. In our stratified analysis, women originally allocated to placebo who subsequently took a calcium supplement in the post-trial period had an increased risk for osteoporotic fracture. Given the inconsistency of this finding within our study data and with other studies, this is likely a chance finding.

Calcium supplements slow the rate of post-menopausal bone loss, but do not prevent it altogether. The offset of the effects of calcium supplements on BMD has been studied previously. Daly *et al.* followed up 109 men over 50 years of age who had completed a 2-year fortified milk trial (providing an additional 1000 mg of calcium and 800 IU of vitamin D_3 per day) after an additional 18 months (656). There were significant differences between the groups at the femoral neck and ultradistal radius, whereas there were no significant differences at the total hip or lumbar spine. Dawson-Hughes *et al*. found a small persisting benefit in total body BMD but not at the femoral neck or lumbar spine in 146 men 2 years after the end of a 3-year randomised controlled trial comparing calcium 500 mg plus 700 IU of vitamin D_3 per day with placebo (299). There were no persisting benefits at the spine, femoral neck or total body for the 167 women studied. In our study, the benefits of calcium on BMD were no longer present 5 years following trial completion. Taken together, the

results suggest that the beneficial effects of calcium supplements on BMD do not persist after the supplements are discontinued.

Calcium supplements increase the incidence of MI and stroke (288,289,657), but the findings of our study, together with another similar study (658), suggest that there is no excess cardiovascular risk once supplements are stopped. The RECORD investigators have published data for total vascular disease, cardiovascular and cerebrovascular disease deaths from their original study (intervention with calcium and/or vitamin D over 24 to 62 months) plus a further three years of post-trial follow-up (659). There were no statistically significant between-group differences for any endpoint. Therefore, it appears that the adverse effects of calcium supplements on cardiovascular risk do not persist after treatment is discontinued.

This study has some limitations. Although it is a follow-up of a randomised controlled trial, the post-trial extension was observational in nature and has the potential for bias inherent in such studies. The number of participants was limited to those women who survived the trial period and the study did not have sufficient power to detect small between-group differences in cardiovascular event rates. Methods of event ascertainment were different for each period of follow-up, with adjudication of self-reported events and events identified from the database searches in the original trial but not during the post-trial follow-up. Fracture events in the trial period were verified, whereas post-trial fracture events were self-reported and supplemented by events identified from the database searches. However, any bias introduced would be expected to be non-differential, and results were similar in sensitivity analyses when events were restricted to those obtained from the national databases for hospital admissions and death. Since information on post-trial medication was obtained at the time of a single telephone interview, we do not have complete information on duration of medication use or dose, nor do we have this information for the entire post-trial cohort. This limited our

ability to extrapolate what effects post-trial calcium use may have had on post-trial cardiovascular events.

In summary, although calcium supplements appear to have small positive effects on total fracture incidence and BMD during treatment, upon withdrawal these effects are not maintained. However, beneficial effects on vertebral fracture and forearm fracture were observed, but these findings need confirmation from other studies before being accepted. In addition, the increased cardiovascular risk with calcium supplements, which outweighs any positive skeletal effects (289,657) does not persist once supplements are stopped.

3. SUBGROUP ANALYSIS FOR THE RISK OF CARDIOVASCULAR DISEASE WITH CALCIUM SUPPLEMENTS.

3.1. Introduction

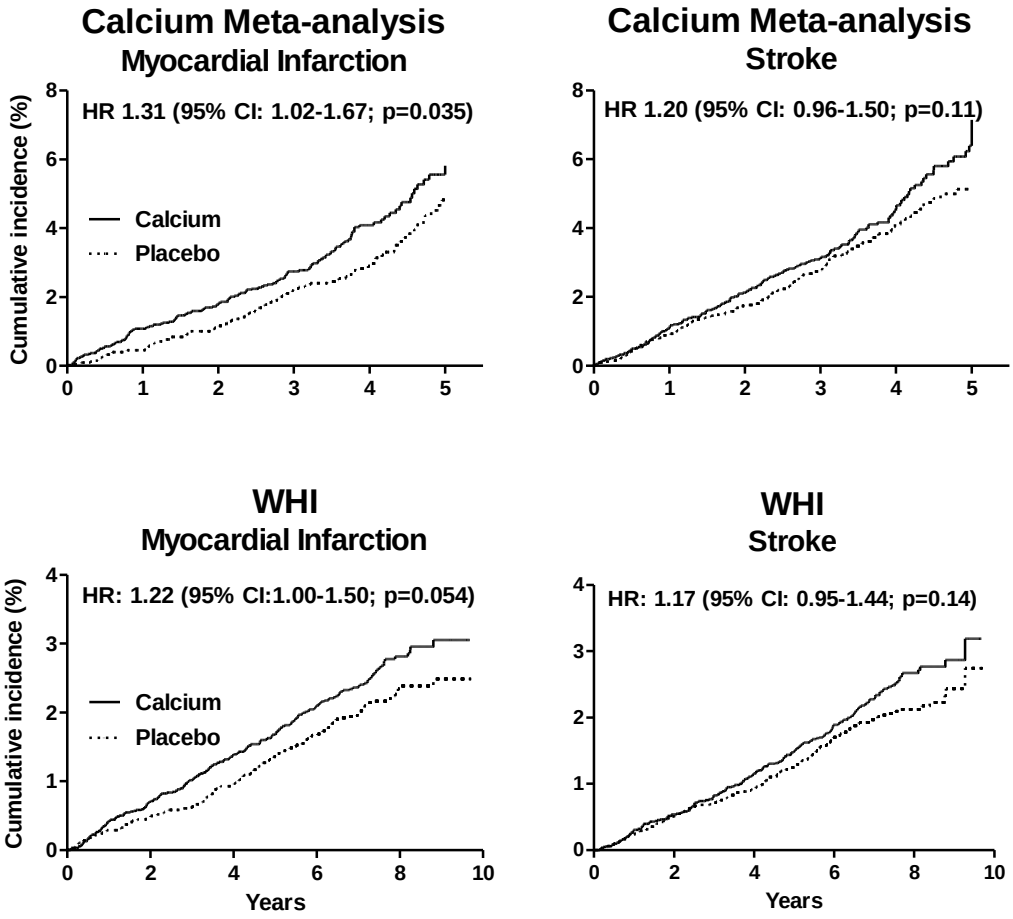
Calcium supplements have been widely used for the treatment and prevention of osteoporotic fractures, but recently their cardiovascular safety has been questioned. A secondary analysis of the Auckland Calcium Study showed a 43% increase in the rate of cardiovascular events in women randomised to 1 g daily calcium (as citrate) (288). In a subsequent meta-analysis of 11 randomised, placebo-controlled trials of calcium supplements with nearly 12,000 participants, calcium increased the risk of myocardial infarction by 27-31% (289). In a re-analysis of the Women's Health Initiative Calcium and Vitamin D study (WHI CaD), calcium co-administered with vitamin (CaD) increased the risk of myocardial infarction (MI) by 22% in women who were not taking personal, non-protocol calcium supplements at randomization (657). Figure 3.1 shows that the results from the meta-analysis of calcium monotherapy and the re-analysis of WHI CaD were strikingly similar, including the longer latency for the development of the effect on stroke. Because of this similarity in outcomes, we pooled the data sets producing a meta-analysis of trials of calcium supplements with or without vitamin D. Thirteen trials qualified for this, involving nearly 30,000 participants. Calcium increased the risk of MI by 25% and stroke by 15-20% (657).

An important question is whether the increased cardiovascular risk from calcium supplements is consistent across the population, or whether some patient groups are at greater risk. There is already some evidence suggesting this. For example, in the meta-analysis of trials of calcium monotherapy, there was an interaction between dietary calcium intake and the risk of MI with calcium supplements (289,660). In the group with dietary calcium intake above the median (805 mg/day) there was an increased risk of MI with calcium supplements, whereas there was no increase in risk in the group with dietary calcium intake below the median,

though these trends were no longer apparent when the analysis was done based on quintiles of dietary calcium intake. In the primary analysis of WHI CaD, there was an interaction between body mass index (BMI) and the risk of MI or death from coronary heart disease (CHD) with an elevated risk of this composite endpoint with CaD only in women with BMI <30 kg/m^2 (362). In a 5-year randomised controlled trial of calcium supplements, Lewis *et al.* reported that calcium supplements reduced the risk of atherosclerotic vascular disease in women with known atherosclerotic vascular disease at baseline (658).

To explore these contrasting findings, we investigated whether the effects of calcium supplements on the risk of MI and stroke vary across different subgroups in our re-analysis of WHI CaD, or in the pooled patient-level dataset of trials of calcium supplements with or without vitamin D. Detailed subgroup analyses have not previously been carried out in these data sets. WHI CaD had a broader range of baseline data than the pooled data set, allowing for a greater variety of subgroups to be assessed.

Figure 3.1 Kaplan Meier survival curves for time to incident myocardial infarction or stroke by treatment allocation in a meta-analysis of patient-level data from 5 trial of calcium supplements used as monotherapy (n=8151) and in women in the Women's Health Initiative calcium and vitamin D (WHI) trial not using personal calcium supplements at randomisation (*n* = 16,718). HR: hazard ratio. CI: confidence interval. (Note the different scales on the y and x axes).



3.2. Methods

In brief, WHI CaD was a randomised, double-blind, placebo-controlled study of 1 g calcium/400 IU vitamin D_3 daily in 36,282 post-menopausal women followed for an average duration of 7 years (362,661). Medical records related to self-reported medical events for MI, stroke, and coronary revascularization were adjudicated centrally by physician adjudicators using standardized definitions, and all deaths were also centrally adjudicated. The primary analysis reported no effect of CaD on cardiovascular events, but 54% of participants were taking personal (non-protocol) calcium supplements at randomization. We obtained the WHI limited-access clinical trials dataset from the National Heart Lung and Blood Institute. In a re-analysis of this dataset, we found interactions between personal calcium supplement use and CaD for cardiovascular events (657). In women not using personal calcium supplements at randomization, CaD increased cardiovascular risk, whereas there was no alteration of risk in women already taking calcium supplements at randomization. We have therefore restricted our current analyses to women not taking personal calcium supplements at randomization.

For the meta-analysis of calcium with or without vitamin D, we searched Medline, Embase and the Cochrane Central Register of Controlled Trials for randomised placebo controlled trials of calcium supplements used as monotherapy in March 2010 (289). Eligible studies were randomised, placebo-controlled trials of calcium supplements (≥500 mg/day), with 100 or more participants of mean age more than 40 years, and study duration of more than one year. 15 trials were eligible, 6 supplied trial-level data only, and 5 supplied patient-level data. In these 5 trials, cardiovascular events were from unadjudicated self-reports (1 study); adjudicated self-reports and death certificates (1 study); verified events from hospital discharge data and adjudicated death certificates (1 study); self- reports, hospital admissions, and death certificates that were independently adjudicated by a cardiologist or neurologist (2 studies). A systematic review identified 2 randomised, placebo-controlled trials of CaD with

cardiovascular outcomes- WHI CaD and another small study (604). We updated the patient-level dataset for trials of calcium monotherapy with our re-analysis of WHI CaD, restricting the dataset to women not using personal calcium supplements at randomization. Thus, the complete dataset comprised 24,869 people in 6 trials, with an average participant age of 66 years, 93% female and average duration of follow-up of 5.9 years. This is the same database used in our previous meta-analysis of calcium with or without vitamin D (657).

For the current analyses of the WHI CaD dataset, we attempted to replicate the approach of the WHI investigators where possible. The baseline characteristics at the time of randomization to CaD are reported, whereas the WHI investigators reported these characteristics at entry to the WHI programme (362). For BMI, dietary and supplemental calcium intake, we used the latest value recorded between screening and one month following CaD randomization. For variables related to medical history, we used the status at entry to the WHI programme. We modelled the effect of CaD on the time to incident MI, stroke, and the composite endpoint of coronary revascularisation (either percutaneous coronary angioplasty or coronary artery bypass grafting) as well as percutaneous coronary angioplasty (PTCA) and coronary artery bypass grafting (CABG) individually in pre-specified subgroups for baseline age (<60, 60-70, >70 years), dietary calcium intake (<500, 500-700, 700-900, 900-1100, ≥1100 mg/day, as well as above and below the median value of 702 mg/day), BMI (<25, 25-30, ≥30 kg/m^2), smoking history, and previous MI or stroke using interaction terms in Cox proportional hazards models stratified by age, prevalent cardiovascular disease at baseline, and randomization status in the WHI hormone and dietary modification trials, following the approach of the WHI investigators (362,661).The dietary calcium thresholds represented the quintile of intake rounded to the nearest 100 mg/d.

In the meta-analysis dataset, we repeated these analyses modelling the effect of treatment allocation (calcium with or without vitamin D) on the time to incident MI and stroke in the

following pre-specified subgroups for baseline age (<60, 60-70, ≥70 years), dietary calcium intake (<500, 500-700, 700-900, 900-1100, ≥1100 mg/day, as well as above and below the median value of 737 mg/day), history of cardiovascular disease, history of smoking , history of diabetes mellitus, and history of hypertension using interaction terms in Cox Proportional Hazard models stratified by study. The assumption of proportional hazards was tested by performing a test for proportionality of the interaction between variables included in the model and the logarithm of time. All analyses were performed using SAS version 9.2. All tests were two tailed and $p < 0.05$ was considered significant.

3.3. Results

Table 3.1 depicts selected baseline characteristics of women in WHI CaD who were not using calcium supplements at randomization. There were no significant differences between the groups. Figures 3.2-3.6 show the interactions between WHI CaD allocation and baseline characteristics for the risk of MI, stroke, coronary revascularisation, PTCA and CABG. For all of these endpoints, we found no evidence of significant interactions between treatment allocation and any of the baseline variables in the complete WHI CaD dataset (362), there was a significant interaction between allocation to CaD, BMI and the composite endpoint of MI or CHD death. In our analysis of women not taking calcium supplements at randomization, we found no statistically significant interactions between allocation to CaD, BMI, and either MI, stroke, coronary revascularisation, PTCA or CABG (Figures 3.2-3.6). As our findings differed from the primary WHI CaD analysis, we repeated our analyses in women using personal calcium supplements at randomization and found a significant interaction between BMI and allocation to CaD for the risk of MI ($p = 0.049$), with the risk of MI from CaD inversely related to BMI (Table 3.2, Figure 3.7). To explore whether these findings were related to difference in baseline characteristics between the subgroups, we adjusted for previous stroke, previous MI, smoking history, diabetes history, age at

randomization, baseline systolic blood pressure and baseline dietary calcium intake but the hazard ratios did not substantially change.

Table 3.1 Selected baseline characteristics of women in the Women's Health Initiative calcium and vitamin D study who were not taking calcium supplements at baseline. Data, other than mean (standard deviation), are %.

Characteristic	CaD (*n* = 8429)	Placebo (*n* = 8289)	*p*-value
Age (y):			
Mean (SD)	62.9 (7.0)	62.9 (7.0)	0.91
<60	39	38	
60-70	43	44	
>70	18	18	
Body Mass Index (kg/m^2)			
Mean (SD)	29.4 (5.9)	29.4 (6.0)	0.80
<25	24	25	
25-30	36	34	
≥30	40	41	
Dietary calcium (mg/d):			
Mean (SD)	804 (489)	798 (475)	0.42
<500	28	29	
500-700	21	21	
700-900	17	18	
900-1100	12	12	
≥1100	21	20	
History of MI	2.3	2.0	0.26
History of Stroke	1.0	1.2	0.35
Smoking history			0.64
Never	51	52	
Previous	39	38	
Current	9	9	

Figure 3.2 Risk of myocardial infarction in women in the Women's Health Initiative calcium and vitamin D trial not using personal calcium supplements at randomization by treatment allocation in subgroups defined by various baseline characteristics. Results are reported as hazard ratios with 95% confidence intervals (CI) (horizontal bar). The dotted vertical line represents the hazard ratio in the entire cohort (HR 1.22, 95% CI 1.00-1.50, $p = 0.05$).

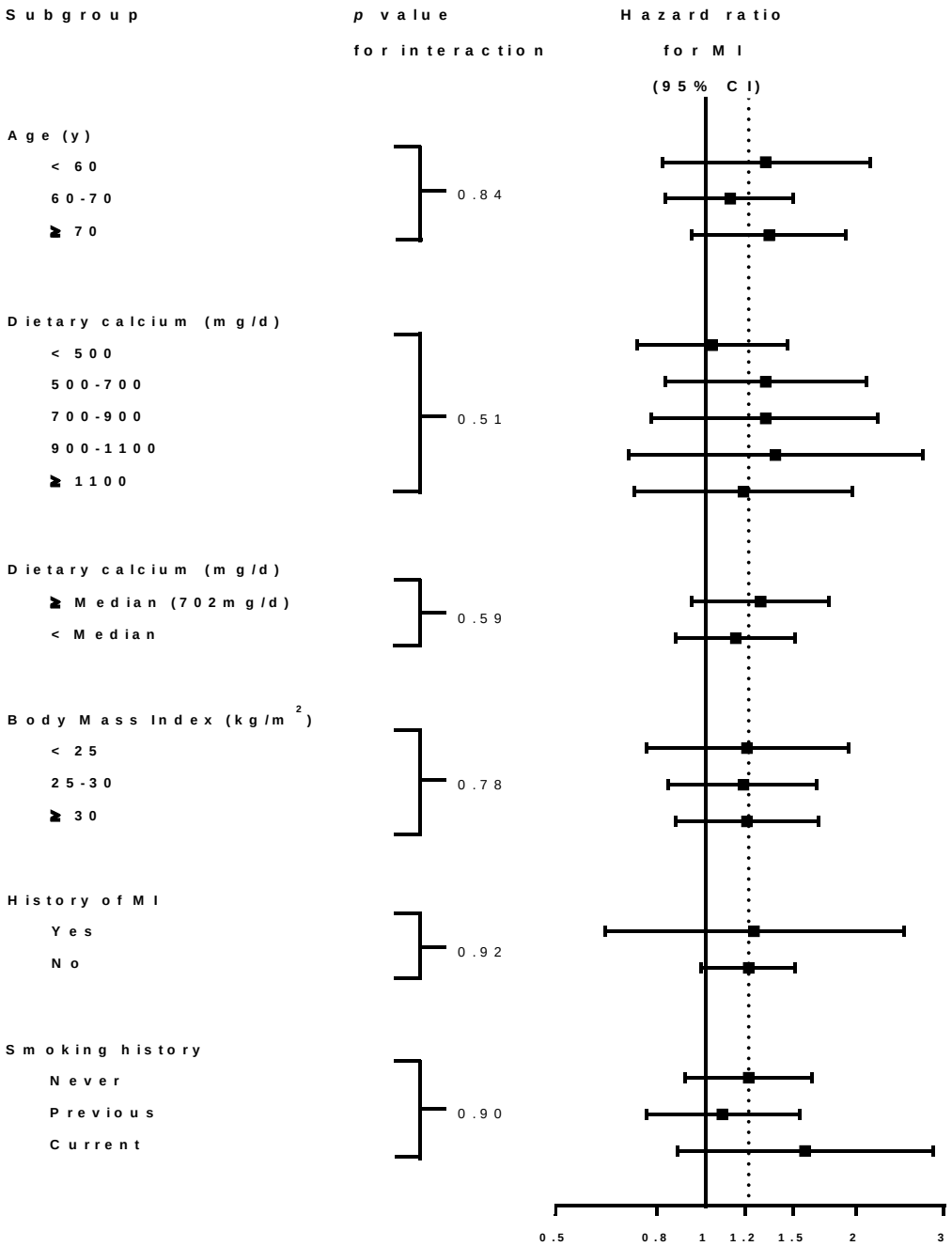


Figure 3.3 Risk of stroke in women in the Women's Health Initiative calcium and vitamin D trial not using personal calcium supplements at randomization by treatment allocation in subgroups defined by various baseline characteristics. Results are reported as hazard ratios with 95% confidence intervals (CI) (horizontal bar). The dotted vertical line represents the hazard ratio for the entire cohort (HR 1.17, 95% CI 0.95-1.44, $p = 0.14$).

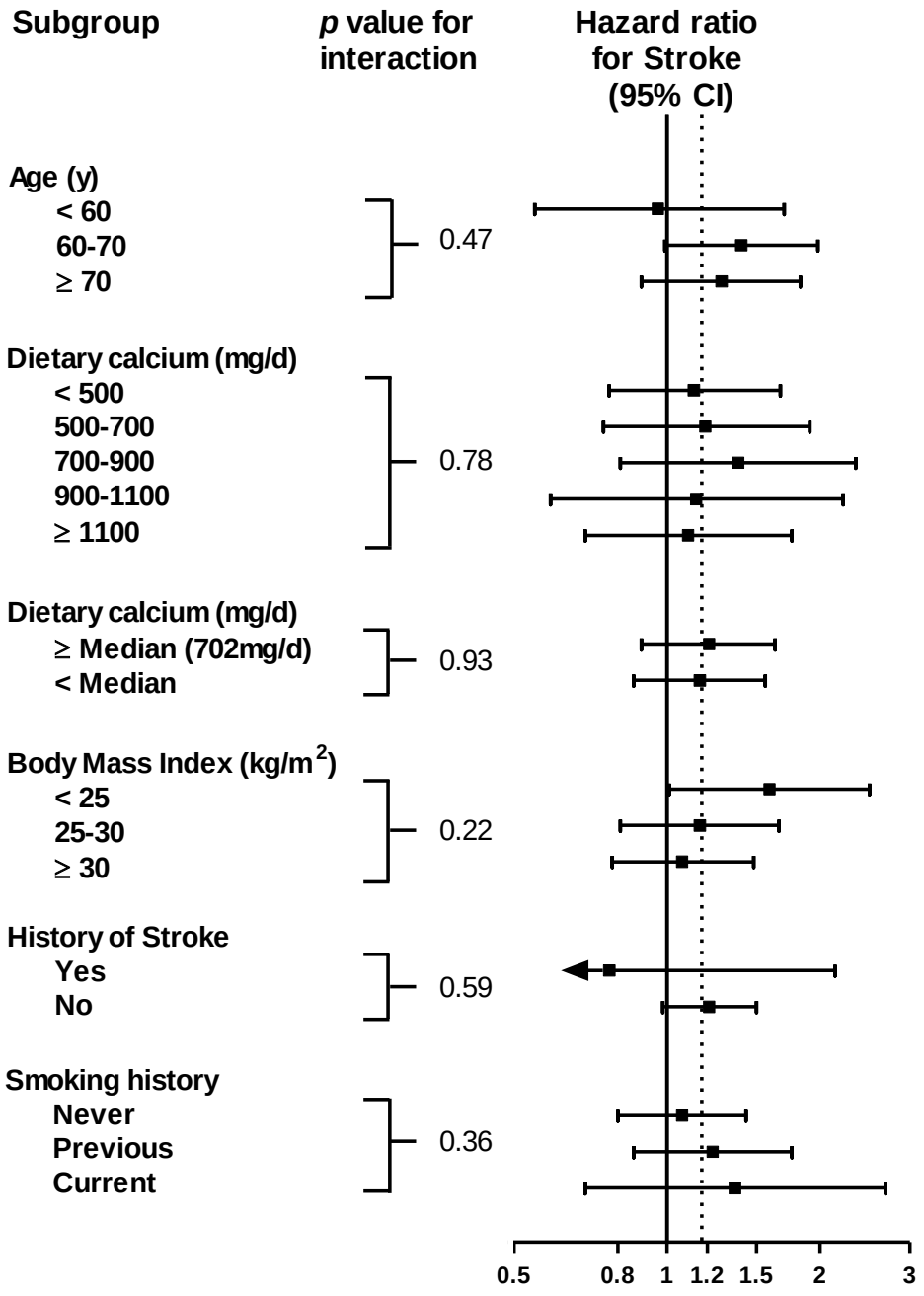


Figure 3.4 Risk of coronary revascularisation in women in the Women's Health Initiative calcium and vitamin D trial not using personal calcium supplements at randomization by treatment in subgroups defined by various baseline characteristics. Results are reported as hazard ratios with 95% confidence intervals (CI) (horizontal bar). The dotted vertical line represents the hazard ratio for the entire cohort (hazard ratio 1.15, 95% CI 0.98-1.34, p = 0.09).

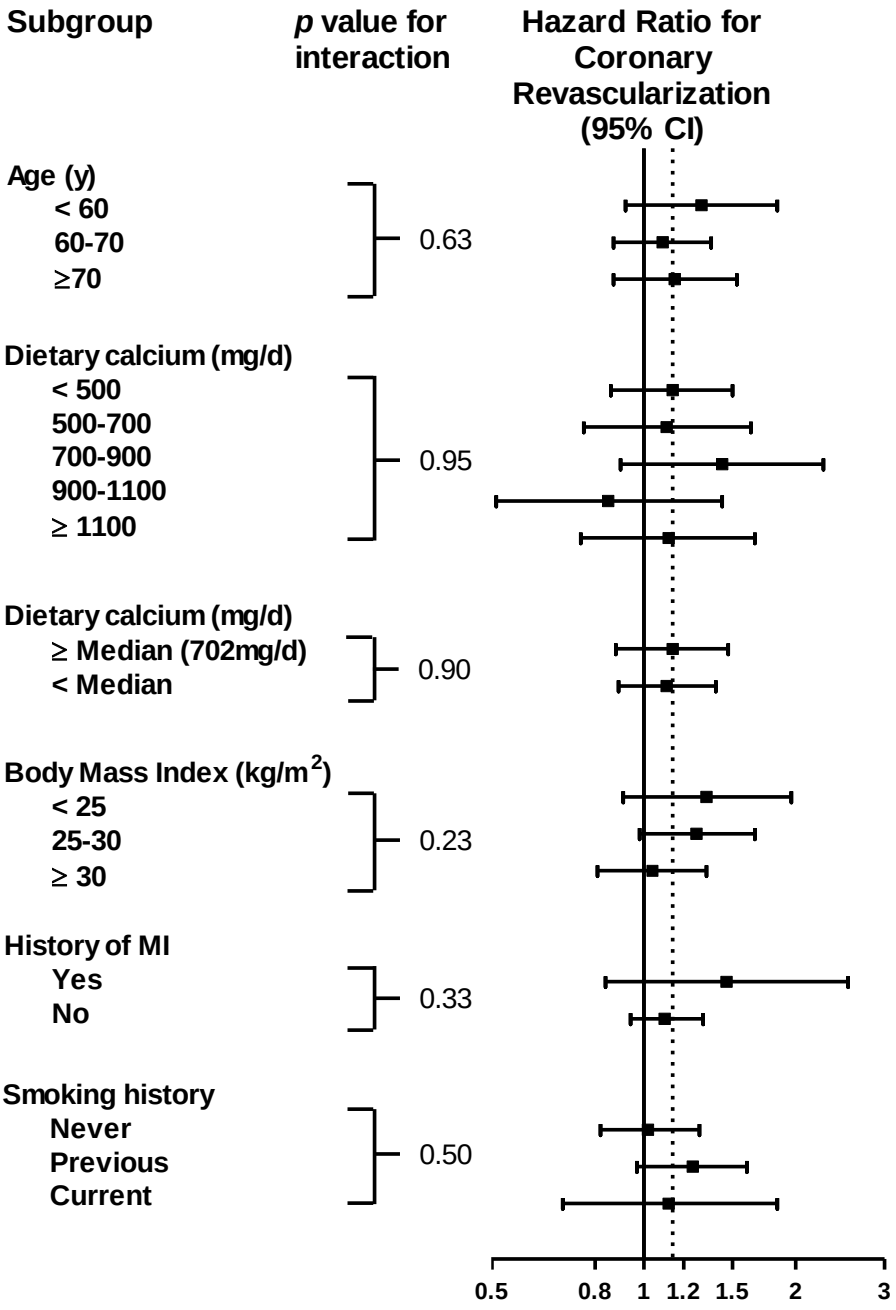


Figure 3.5 Risk of Coronary Artery Bypass grafting (CABG) surgery in women in the Women's Health Initiative calcium and vitamin D trial not using personal calcium supplements at randomization by treatment allocation in subgroups defined by various baseline characteristics. Results are reported as hazard ratios with 95% confidence intervals (CI) (horizontal bar). The dotted line represents the hazard ratio in the entire cohort (hazard ratio 1.36, 95% CI 1.06-1.75; $p = 0.017$).

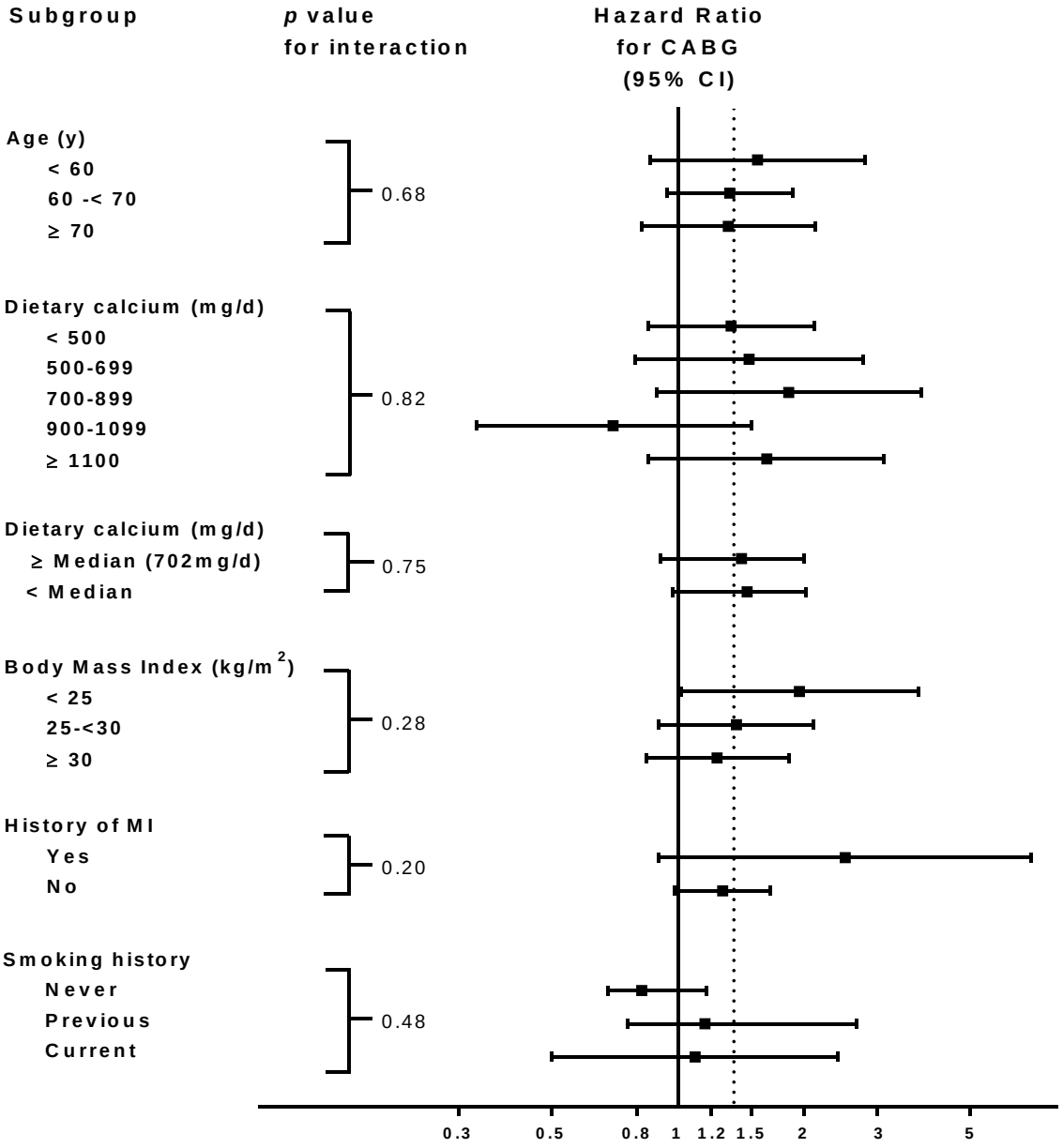
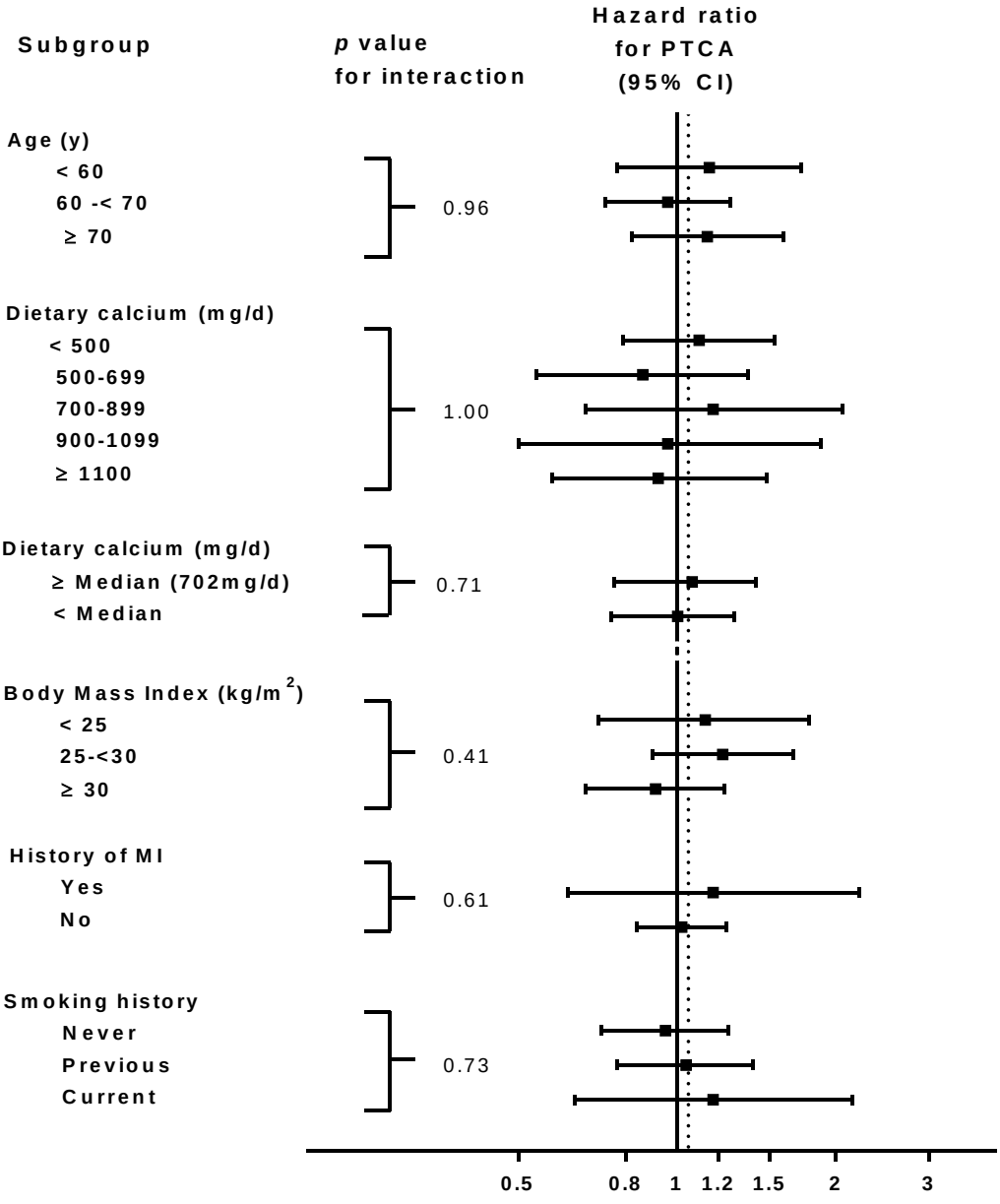


Figure 3.6 Risk of Percutaneous transluminal coronary angioplasty (PTCA) in women in the Women's Health Initiative calcium and vitamin D trial not using personal calcium supplements at randomization by treatment allocation in subgroups defined by various baseline characteristics. Results are reported as hazard ratios with 95% confidence intervals (CI) (horizontal bar). The dotted line represents the hazard ratio in the entire cohort (hazard ratio 1.05, 95% CI 0.87-1.26; $p = 0.63$).



In the pooled patient-level meta-analysis dataset, the overall hazard ratio for time to incident MI for calcium with or without vitamin D was 1.25 (95% CI 1.06-1.46; $p = 0.0065$) and for time to incident stroke was 1.19 (95% CI 1.02-1.39; $p = 0.026$). There were no significant interactions between treatment allocation and age, gender, dietary calcium intake, history of cardiovascular disease, smoking history, diabetes mellitus, or hypertension either for the risk of MI (Table 3.3), or for the risk of stroke (Table 3.4).

Figure 3.7 Effect of body mass index on the risk of myocardial infarction in women in the Women's Health Initiative calcium and vitamin D trial who did and did not use personal calcium supplements at randomization by treatment allocation. Results are reported as hazard ratios with 95% confidence intervals (CI) (horizontal bar). The dotted vertical line represents the hazard ratio for women who were not using calcium supplements at randomization (HR 1.22; 95% CI 1.0,1.5; $p = 0.054$). The dashed vertical line represents the hazard ratio for women who were using calcium supplements at randomization (HR 0.92; 95% CI 0.75,1.13; $p = 0.44$).

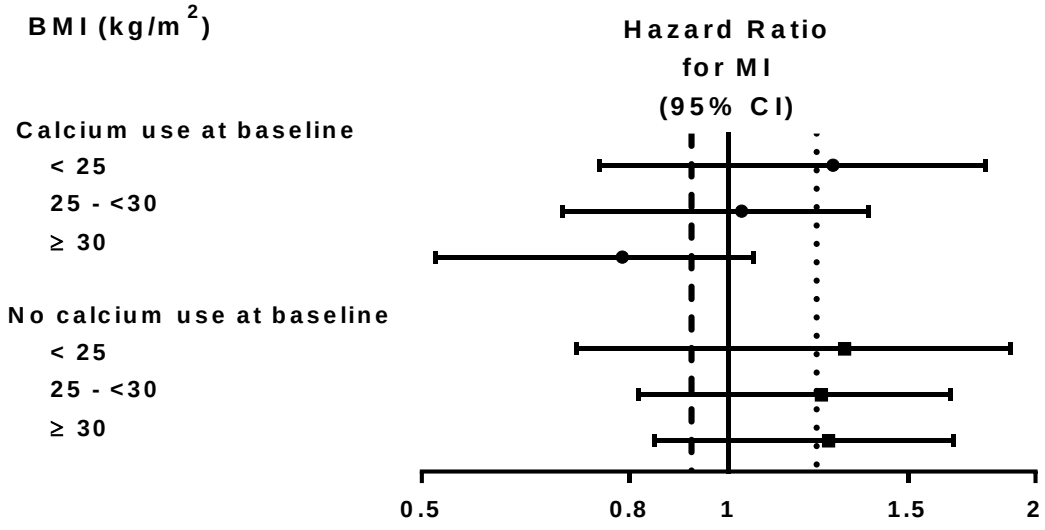


Table 3.2 Effect of body mass index on the risk of myocardial infarction and stroke with calcium/vitamin D in the Women's Health Initiative calcium and vitamin D Study, grouped by personal use of calcium supplements at randomisation.

	No personal use of calcium				Any personal use of calcium			
	CaD *n* (%)	Placebo *n* (%)	HR (95% CI)	*p*-value for interaction	CaD *n* (%)	Placebo *n* (%)	HR (95% CI)	*p*-value for interaction
MI				0.78				0.049
BMI<25	39 (1.9)	34 (1.6)	1.21 (0.76-1.93)		48 (1.6)	42 (1.3)	1.19 (0.79-1.82)	
BMI 25-30	75 (2.5)	61 (2.1)	1.19 (0.84-1.67)		72 (2.1)	71 (2.0)	0.99 (0.71-1.39)	
BMI ≥30	95 (2.8)	73 (2.2)	1.21 (0.87-1.68)		60 (1.8)	83 (2.6)	0.76 (0.53-1.07)	
Stroke				0.22				0.73
BMI<25	48 (2.4)	33 (1.6)	1.59 (1.01-2.49)		47 (1.5)	58 (1.8)	0.83 (0.56-1.23)	
BMI 25-30	68 (2.3)	56 (2.0)	1.16 (0.81-1.66)		62 (1.8)	73 (2.1)	0.88 (0.62-1.24)	
BMI ≥30	80 (2.4)	74 (2.2)	1.07 (0.78-1.48)		47 (1.4)	58 (1.8)	0.78 (0.52-1.15)	

3.4. Discussion

In women in WHI CaD who were not taking calcium supplements at baseline and in the pooled patient-level meta-analysis, we found no evidence for interactions between calcium supplements (with or without vitamin D) and age, gender, BMI, baseline dietary calcium intake, smoking status, previous history of cardiovascular disease, diabetes mellitus, or history of hypertension for the risk of MI, stroke, or coronary revascularisation.

Previously, in a meta-analysis of 5 studies of calcium monotherapy, we reported a significant interaction between dietary calcium intake and the risk of MI with calcium supplements (289). The group with intake greater than the median of 805 mg/day had an increased risk of MI with calcium, whereas those with intake below the median had no alteration of risk. However, when the cohort was divided by quintile of dietary calcium intake, there was no evidence of a dose-response relationship. There was also no interaction between dietary calcium intake and the risk of stroke, or the composite cardiovascular endpoint with calcium.

Table 3.3: Risk of myocardial infarction by treatment allocation in subgroups in patient-level meta-analysis dataset.

	Calcium/CaD n (%)	Placebo n (%)	HR (95% CI)	*p*-value for interaction
Age (y)				0.62
<60	45 (1.2)	32 (0.9)	1.28 (0.81-2.02)	
60-70	108 (2.7)	95 (2.4)	1.10 (0.83-1.46)	
≥70	199 (4.1)	152 (3.1)	1.37 (1.10-1.70)	
Gender				0.73
Male	38 (4.0)	32 (3.8)	1.22 (0.73-2.06)	
Female	314 (2.7)	247 (2.2)	1.28 (1.08-1.52)	
Dietary calcium (mg/d)				0.39
<400	63 (3.1)	59 (3.1)	1.19 (0.81-1.74)	
400-600	80 (3.1)	74 (2.8)	1.05 (0.76-1.46)	
600-800	63 (2.6)	45 (1.9)	1.52 (1.01-2.28)	
800-1100	81 (2.8)	52 (1.9)	1.24 (0.85-1.80)	
≥1100	65 (2.5)	49 (1.9)	1.32 (0.89-1.94)	
Dietary calcium (mg/d)				0.10
≥ Median (737mg/d)	164 (2.6)	117 (1.9)	1.31 (1.02-1.67)	
< Median	188 (3.0)	162 (2.6)	1.23 (0.99-1.53)	
History of CVD				0.32
Yes	50 (4.7)	49 (4.6)	1.03 (0.69-1.53)	
No	133 (2.1)	100 (1.6)	1.31 (1.01-1.70)	
Smoking history				0.89
Never	126 (2.5)	103 (2.1)	1.20 (0.92-1.57)	
Previous	93 (2.4)	74 (2.0)	1.14 (0.83-1.57)	
Current	49 (4.0)	37 (3.2)	1.39 (0.88-2.18)	
Diabetes mellitus				0.18
Yes	66 (7.8)	40 (4.9)	1.74 (1.15-2.65)	
No	285 (2.4)	239 (2.1)	1.19 (1.00-1.42)	
History of hypertension				0.17
Yes	132 (4.1)	102 (3.1)	1.40 (1.07-1.82)	
No	128 (1.9)	102 (1.6)	1.06 (0.81-1.38)	

CaD- calcium and vitamin D, HR- hazard ratio, CI- confidence interval.

Therefore, we concluded that the evidence for a relationship between dietary calcium intake, calcium supplement use, and cardiovascular risk was only weak. The current study supports this conclusion: there was no significant interaction between dietary calcium intake (both when grouped by median intake and by quintiles of intake) and allocation to calcium with or without vitamin D for the risk of MI, stroke, or coronary revascularisation in our re-analysis of WHI CaD or the pooled patient-level meta-analysis dataset.

Table 3.4: Risk of Stroke by treatment allocation in various subgroups in patient-level meta-analysis dataset.

	Calcium/CaD n (%)	Placebo n (%)	HR (95% CI)	*p*-value for interaction
Age (y)				0.57
<60	25 (0.7)	24 (0.7)	1.01 (0.58-1.77)	
60-70	102 (2.5)	87 (2.2)	1.19 (0.89-1.58)	
≥70	236 (4.8)	195 (4.0)	1.22 (1.01-1.48)	
Gender				0.47
Male	31 (3.2)	31 (3.7)	1.00 (0.61-1.65)	
Female	332 (2.9)	275 (2.4)	1.22 (1.04-1.43)	
Dietary calcium (mg/d)				0.67
<400	55 (2.7)	47 (2.4)	1.12 (0.76-1.66)	
400-600	72 (2.8)	70 (2.6)	1.06 (0.76-1.47)	
600-800	69 (2.9)	44 (1.8)	1.59 (1.09-2.33)	
800-1100	91 (3.1)	86 (3.1)	1.05 (0.78-1.41)	
≥1100	76 (2.9)	59 (2.3)	1.31 (0.94-1.85)	
Dietary calcium (mg/d)				0.90
≥ Median (737mg/d)	189 (3.0)	160 (2.6)	1.21 (0.98-1.49)	
< Median	174 (2.8)	146 (2.3)	1.18 (0.95-1.47)	
Smoking history				0.34
Never	123 (2.4)	112 (2.2)	1.13 (0.87-1.45)	
Previous	88 (2.3)	66 (1.8)	1.28 (0.93-1.76)	
Current	46 (3.8)	33 (2.9)	1.49 (0.78-2.86)	
Diabetes mellitus				0.070
Yes	39 (4.6)	44 (5.3)	0.82 (0.53-1.27)	
No	324 (2.8)	262 (2.3)	1.26 (1.07-1.48)	
History of hypertension				0.41
Yes	121 (3.8)	107 (3.3)	1.16 (0.89-1.50)	
No	123 (1.9)	89 (1.4)	1.37 (1.04-1.80)	

CaD- calcium and vitamin D, HR- hazard ratio, CI- confidence interval.

In the primary analysis of WHI CaD, Hsia et al reported a significant interaction between BMI and the use of CaD for the risk of the composite endpoint of MI or CHD death, with an increased risk with CaD observed in women with BMI <30 kg/m^2 (362) . In contrast, we observed no interaction between CaD and BMI for the risk of MI in women in WHI CaD who did not use calcium supplements at randomization. However, in those women using non-protocol calcium supplements at randomization, there was a significant interaction between BMI, CaD, and the risk of MI, with an inverse relationship between BMI and the risk of MI

from CaD. In women with normal BMI (<25 kg/m^2), the hazard ratio was 1.19, similar to the risk observed in women not taking personal calcium supplements at randomisation, whereas overweight and obese women had no alteration of risk (hazard ratio 0.99) and a reduced risk (hazard ratio 0.76) respectively. This inverse relationship persisted after adjustment for traditional cardiovascular risk factors.

Lewis and colleagues found that calcium supplements reduced the risk of an atherosclerotic vascular event during 5 years of follow up for women who had a history of atherosclerotic vascular disease (658). This result should be treated with great caution for several reasons. The composite outcome contained endpoints that may result from a wide number of pathogenetic processes unrelated to atherosclerosis, such as atrial fibrillation and congestive heart failure. All patient events were obtained from unadjudicated hospital discharge codes, and only the primary code for each admission was utilised, which is likely to have resulted in missed events. For example, there were 28 MIs identified from coding in 1460 women of mean age 75 years followed for 5 years. Compared to other studies in our meta-analysis of calcium monotherapy (289), this event rate was approximately half to one third the rate in females of similar age in two studies (650,653), and similar to the rate in females of mean age 59 years in one study (605) and 63.5 years in another study (362).The study lacked adequate power, either in the primary analysis or in subgroup analyses, to detect differences in event rates between the treatment groups of the magnitude observed in our meta-analyses. Finally, the authors have not followed recommended guidance for the reporting of subgroups (620,640), in that they have reported hazard ratios and *p* values for single subgroups. The recommended approach is to report the results of interaction tests between subgroups (i.e. the subgroups with or without atherosclerotic vascular disease at baseline) and only consider individual subgroup results if the interaction test is statistically significant. We did not confirm interactions between history of MI, or stroke and the risk of cardiovascular events

with calcium in WHI CaD. Thus, the most likely explanation for the finding by Lewis and colleagues is chance.

Our study has some limitations. As we used the WHI limited-access clinical trials dataset for our analysis, we are limited to the information available in this dataset. Subgroup analyses introduce limitations with the possibility of falsely significant results and therefore significant results require cautious interpretation. However, we have not identified significant interactions in the current analysis. Lack of power is also potentially an issue when performing subgroup analyses, because the decrease in the number of relevant events in each group analysed may result in a Type 2 error. The large number of events in the dataset suggests that if such an error occurred it is not likely to be clinically relevant.

In conclusion, calcium supplements with or without vitamin D are associated with an increased risk for MI and stroke, and this risk appears constant across subgroups defined by important baseline characteristics. Thus, we have found no evidence that the increased cardiovascular risk from calcium supplements differs within patient populations.

4. EFFECTS OF CALCIUM SUPPLEMENT INTAKE ON MOUSE SERUM MINERAL PROFILE AND GENE EXPRESSION

4.1. Introduction

Calcium supplements are associated with an increased risk for cardiovascular disease. Evidence to support this arises from large prospective, observational studies (337,662); from secondary analyses of randomised controlled trials (288); and from meta-analyses of randomised controlled trials (289,657). When considering patient level data, the increased risk for myocardial infarction is 26% (95% confidence interval CI 1.07-1.47; $p = 0.005$) and for stroke is 19% (95% CI 1.02 to 1.39; $p = 0.03$). Body mass index (362), dietary calcium intake (289,363) and previous cardiovascular disease (658) were thought to modulate the effect of calcium supplements on cardiovascular disease but a subgroup analysis (663) found that many important baseline characteristics do not alter this increased risk.

Calcium supplements may cause an increase in cardiovascular disease through their ability to increase serum calcium (312,320,321,326,664). Elevated serum calcium levels as well as levels at the upper limit of normal are associated with cardiovascular disease (344,346,351,352,354,665), metabolic syndrome (666), impaired glucose tolerance (667) and increased carotid artery thickness (668), also itself associated with cardiovascular disease. Hypercalcaemia may exert a detrimental effect on cardiovascular disease by increasing vascular calcification, causing blood hypercoagulability (228), altering vessel vasodilatory response (669), or in other ways yet to be determined.

Although much remains to be uncovered, experiments involving vascular smooth muscle cell (VSMC) cultures provide insights into the pathogenesis of vascular calcification and the role calcium may play therein. Vascular calcification is the pathological process of calcium deposition in the vascular intima and or media as calcium phosphate. Once thought to be a

passive process whereby an elevated calcium-phosphorus product resulted in spontaneous precipitation, this is no longer accepted as true with a plethora of data providing evidence for an orchestrated system created to minimize harm (670). It is the result of one or a combination of the following: 1) the production of mineralizing competent matrix vesicles released from vascular cells, 2) apoptosis of vascular smooth muscle cells, 3) a phenotypic trans-differentiation of vascular smooth muscle cells and other cells of mesenchymal origin (e.g. pericytes) to an osteogenic/chondrogenic phenotype, or 4) the loss of mineralizing inhibitors due to a gene mutation or from over consumption. Thereafter, crosstalk acts to stoke mineralization. This contrasts with the biologically normal process of mineralization that culminates in the formation of teeth and bone.

Intimal calcification is associated with age and the cumulative atherosclerotic inflammatory damage observed throughout the vasculature that plays a role in vessel narrowing and subsequent myocardial infarction and stroke. Here repetitive inflammatory insults trigger vascular smooth muscle cells to transdifferentiate into an osteochondrogenic phenotype thereby substituting a cell that is highly reactive to inflammatory signals to a relatively inert one. Calcification of the coronary arteries and aorta (671,672) is correlated with poor cardiovascular outcomes. In addition, valvular calcification is associated with valve failure and subsequent morbidity and mortality (458,459).

Medial calcification (also known as Monckeburg sclerosis or arteriosclerosis) is evident in chronic kidney disease and patients with diabetes mellitus, often as sheets of calcification within the tunica media. The vessel wall rids itself of excess calcium and phosphate by sequestering them into matrix vesicles that contain inhibitors of calcium crystal formation such as Fetuin-A. This is followed by cell exocytosis. Fetuin-A is synthesized in the liver and circulates in blood with increases in local calcium concentration signalling cell endocytosis. With time the cells supplies are exhausted resulting in a matrix vesicle that has lost its

inhibitory function becoming calcification competent and therefore apatite formation ensues. This leads to destruction of elastin fibres through the production of matrix metalloproteinases (545,673) and therefore abolition of vessel elasticity and loss of the Windkessel effect culminating in cardiac and peripheral ischaemia as well as hypertension, left ventricular overload and subsequent left ventricular failure. Furthermore, the excess cardiovascular death observed in patients with end-stage renal disease appears in part to be associated with vascular calcification burden. The two forms of calcification are not mutually exclusive.

Adiponectin, a hormone derived predominantly from adipocytes, was first identified in 1995 (600) when four groups sought the hormone simultaneously in both humans and mice. Unlike the discovery of leptin, the explosion in subsequent research did not occur until later that decade when the association between adiponectin and aspects of the metabolic syndrome were observed. We now know that adiponectin has a plethora of actions playing important roles in cellular energy pathways as well as displaying both anti-inflammatory and anti-apoptotic features. It is one mechanism by which adipose tissue exerts direct control over vascular function. Although it is secreted predominantly by adipocytes, adiponectin is made in many other tissues including the myocardium. It is synthesized as a single polypeptide (monomer) but undergoes multimerization within the endoplasmic reticulum to be secreted into the circulation as trimers (low molecular weight form), hexamers (medium molecular weight form) and high molecular weight forms of 18-mers and larger in humans. These multimers appear to have cell specific interactions and therefore actions. Insulin resistance, diabetes mellitus (604,605) and family history of diabetes mellitus (674), obesity (605,609), coronary heart disease (604,611) and stroke are associated with reduced levels of adiponectin as is the male gender (604,615,675). Interestingly, this reduction is seen predominantly in the high molecular weight fraction of the ratio of high molecular weight form to total adiponectin as opposed to total adiponectin levels (601,675,676). Increasing serum adiponectin levels

either by weight loss (604,620), adiponectin therapy in animal models of diabetes mellitus (676), or pharmacologically (601) appears to reduce the risk for obesity-related complications. It not only prevents the production of many pro-inflammatory cytokines but also decreases the cellular reactivity to circulating inflammatory chemokines.

Luo *et al*. describe the adiponectin knockout mouse as having a propensity for early vascular calcification (677) which seems biologically plausible given adiponectin's anti-inflammatory and anti-apoptotic effects. Given that the post-menopausal state has been described as the perfect inflammatory storm with an increase in pro-inflammatory cytokines (678-681), the adiponectin knockout mouse appears to be a potential mouse model for studying age-induced vascular changes observed in postmenopausal women who are often prescribed calcium supplements to prevent osteoporotic-related fractures.

Therefore, aiming to take a step closer to understanding the mechanism whereby calcium supplements increase cardiovascular risk we investigated whether calcium supplements increased vascular calcification, altered the serum profile and produced changes in gene expression of vascular cells in a mouse model of accentuated inflammation. We were also interested as to whether providing calcium over 24 hours would mitigate any negative effects of a single calcium bolus.

4.2. Methods

4.2.1. Mice

Adiponectin knockout mice on a C57Bl/6J background were a gift from Baylor College of Medicine, Texas, United States of America. Adiponectin knockout was confirmed with genotyping of DNA extracted from mouse tail-clips and also by confirming absence of gene expression in adipose tissue. At 11 weeks of age 38 male mice were randomised into two treatment groups (calcium jelly and placebo jelly) based on weight. Animals were housed and

fed in the Vernon Jansen Unit of the University of Auckland and all study procedures were approved by the local animal ethics committee. For 32 weeks mice were housed in cages with littermates where they had access to normal chow (1.01% calcium) and water ad libitum. For 5 days a week (Monday through to Friday) each mouse was placed in an individual cage for 2 hours where they were provided a single jelly with or without calcium according to treatment allocation. Chow and water were refreshed daily as required and mice were monitored for adverse effects according to local protocol.

Normal food intake for a mouse is ~5 g chow per day. Normal chow contains 1.01% calcium. Each 1.2 ml calcium jelly contained 120 mg of elemental calcium in the form of calcium carbonate (Appendix 1). Here we aimed to mimic calcium supplement ingestion in humans by providing calcium at a dose three times the normal intake. Jellies were prepared at the start of each week and kept refrigerated.

4.2.2. Tissue retrieval

At the completion of 32 weeks each mouse was weighed prior to undergoing myocardial puncture and blood aspiration under isoflurane anaesthesia. Hearts and aortas were procured for analysis. Tissue for RNA analysis was placed in RNAlater® for stabilization of RNA material. Specimens for histological examination were placed in 10% neutral buffered formalin.

Using a dissecting microscope, the heart was dissected with the aorta intact to the iliac bifurcation. The ascending aorta was then transected from the heart as close as possible to its origin and the aorta was flushed with phosphate buffered saline (PBS). The aorta was then separated into three segments: arch of aorta with transection just distal to the left subclavian artery, thoracic aorta with transection at the diaphragm, and the remaining abdominal aorta. The heart was transected just caudal to the valves with the proximal portion of the heart

placed in formalin for histological examination. A segment of each of the right and left ventricle of the remaining portion of cardiac tissue was retrieved for RNA analysis. Care was taken to stay clear of the septum to ensure biopsies were of said origin.

4.2.3. Serum analysis

Serum was analysed for calcium, albumin, phosphate and magnesium in the LabPLUS facility of Auckland City Hospital. Calcium was corrected for albumin (measured calcium + [(40 – Albumin) x 0.02] and is accurate for albumin levels in the range of 25-50 g/L. All tests were performed on the Roche Cobas 8000 modular platform with calcium, albumin and phosphate run on the c702 module and magnesium run on the c502 module.

4.2.4. Protein quantification

Protein was quantified using the BioRad Protein Assay and was performed as per the manufacturer's instructions for a Standard Microtitre plate using IgG for preparation of the standards. This method was chosen as it performed well in the presence of formic acid that was necessary for calcium extraction.

4.2.5. Calcium quantification

The calcium content of mouse cardiac and aortic tissue was measured using an Inductively-Coupled Plasma Mass Spectrometer (ICP-MS) in the LabPLUS facility of Auckland City Hospital and corrected for protein content. Tissue was dissected and flushed with PBS and dried at 55°celsius. Calcium was extracted using 10% Formic Acid at a 20:1 volume to weight ratio for 7 days at 4°celsius. The supernatant was then prepared for measurement on the ICP-MS as a 1:80 dilution. Samples were prepared in triplicate unless there was insufficient specimen volume in which case they were prepared in duplicate. Results were averaged.

Calcium quantification was performed on the Varian 820-MS ICP Mass Spectrometer and analysed using ICP-MS Expert software. Calibrations were performed as per protocol covering the range 5-250 parts per billion (μg/L) and is linear over this range with a correlation coefficient of >0.999. Within this system 0.16 μg/ml is the lower detection limit for calcium and Scandium 45 is used as an internal standard. The intra-assay and inter-assay inaccuracies, measured as the variation coefficient were below 10%.

4.2.6. Histology

Heart and aortic specimens were initially placed in 10% neutral buffered formalin followed by immersion in 70% ethanol prior to paraffin embedding. Sections were cut at 5μm thickness and then stained for anatomy and visualization of mineralization.

4.2.6.1. Alizarin stain

Slides were dewaxed and dried before staining with 1% Alizarin Red S solution for 5 minutes. Alizarin Red S was adjusted to pH 4.1-4.3 with 10% ammonium hydroxide. They were then rinsed and dehydrated with 100% Ethanol, clearing with xylene and mounting in DPX (Sharlau).

4.2.6.2. Von Kossa stain

Slides were dewaxed and hydrated in water before staining in 1% Silver nitrate under ultraviolent light for 20 minutes. They were then rinsed before immersion in 5% aqueous thiosulphate for 5 minutes. Following a further rinse, they were then counterstained with 0.25% Safranine for 5 minutes prior to rinsing and dehydrating in 100% Ethanol, clearing with xylene and mounted in DPX (Sharlau).

4.2.6.3. Haematoxylin and eosin stain

Sections were deparaffinized in two changes of xylene, cleared in 100% Ethanol and hydrated in tap water before staining with Gills 2 haematoxylin (Surgipath) for 5 minutes. After rinsing they were differentiated with 1% acid alcohol briefly and rinsed prior to staining with Eosin (Surgipath) before dehydrating with 100% Ethanol, clearing with xylene and mounting in DPX (Sharlau).

4.2.7. Gene expression

RNA was purified from mouse tissue and analysed for pre-specified genes implicated in the phenotypic transformation of vascular smooth muscle cells into those of an osteochondrocytic potential as well as local inhibitors, calcium transport and matrix degradation, known associates of vascular calcification.

RNA integrity was preserved at the time of dissection by placing mouse tissue in RNAlater® (Qiagen^{TM}). It was then stored at 4°celsius for 24 hours before being frozen at – 80°celsius. At the time of processing, tissue was removed from – 80°celsius and homogenized in 350 μl of RLT buffer with β-ME (1 ml Buffer RLT + 10 μl β-ME) using a TissueRuptor® (QIAGEN^{TM}) in a 5 ml glass tube for 20 seconds at a time followed by rest on ice. Between specimens the probe was rinsed with distilled water and buffer RLT to avoid contamination. The process was repeated to ensure complete tissue homogenization.

RNA was extracted using the Rneasy® Mini Kit (QIAGEN ^{TM}) with Proteinase K as is recommended in the fibrous tissue protocol from Qiagen ^{TM} for the extraction of RNA from heart and aortic tissue using QIAcube (QIAGEN ^{TM}). RNA purity and concentration were analysed using the NanoDrop 2000 (ThermoFisher Scientific), and RNA stored at -20°celsius.

Complementary DNA (cDNA) was generated by reverse transcription of mouse heart and aortic mRNA with SuperScript™ III (ThermoFisher Scientific), according to the manufacturer's protocol. 96-well plates were manually loaded with cDNA diluted to a concentration of 1:20 and frozen at -20°celsius until required for gene amplification. The same dilution plate was used for each gene analysed. Reverse transcription polymerase chain reactions (RT-PCR) were prepared using Eppendorf ep*Motion*5070 and amplified in ABI Prism 7900HT (Applied Biosystems). TaqMan probes *Runx2*, Pit-1 transporter (*Scl20a1*), Matrix metalloproteinase 2 (*Mmp2*), Annexin A6 (*Anxa6*), Matrix Gla Protein (*Mgp*), Ectonucleotide pyrophosphatase/phosphodiesterase 1 (*Enpp1*), Alkaline phosphatase (*Alpl*), Bone Gla Protein (*Bglap*), Collagen type I alpha 1 (*ColIa1*), Bone sialoprotein (*Ibsp*) were obtained from Applied Biosystems. Quantification of gene expression relative to 18S rRNA was calculated using the *ΔΔ*Ct method according to the manufacturer's instructions (Applied Biosystems). SDS version 2.3 was used for plot analysis.

4.2.8. Statistics

4.2.8.1. Method for weight analysis over time between groups

Between-group differences in mice weights over the course of the study were analysed using a mixed model two-way analysis of variance (ANOVA) to time and treatment main effects and their interaction. Pre-specified comparisons between treatment groups were made at each time point. The overall pairwise error rate of 5% was protected using false discovery rate (FDR) method.

4.2.8.2. Method for serum analysis

Serum samples were taken at study completion prior to sacrifice. Between-group serum analyses were performed using Student's *t*-test. The assumptions of normality and homogeneity of variance were tested.

4.2.8.3. Justification of sample size for calcium deposition

Calcium deposition in the aorta was our primary endpoint for which there was no supporting literature to guide power calculations. Luo *et al.* found an 8 μg of calcium per mg of protein difference in deposition when comparing adiponectin knockout with wild type mice but we had nothing to indicate how much calcium may be deposited by increasing the calcium intake either by a bolus jelly or a high calcium diet in adiponectin mice alone. We pragmatically chose 11 mice for calcium measurement.

At 80% power at the 5% significance level two groups of 11 mice could detect a difference in calcium deposition of 1.3 standard deviations between groups. The standard deviation remains similar (SD 1.32) between two groups of 10 mice. This is a large difference likely to be of scientific interest.

4.2.8.4. Method for analysis of calcium deposition

To understand whether calcium treatment influenced calcium deposition we analysed between group differences using a mixed model two-way ANOVA to site (aorta, heart) and allocation main effects and their interaction. An overall 5% significance level was preserved for prespecified analysis of between group differences at each site by calculating a false discovery rate (FDR) protected *p*-value. This method of multiple comparisons adjustment is appropriate for an exploratory study with further evaluation of "candidate positives" in a follow-up study.

4.2.8.5. Sample size justification for candidate gene analysis

Our secondary analyses comprised gene analyses and histology for descriptive purposes. Within the structural confinements of this study, we predicted from previous experience that

six mice would be a large enough sample size to give an indication of any treatment effect. Samples of this size typically enable fold changes in excess of two to be detected.

4.2.8.6. Method for candidate gene analysis

To understand whether calcium treatment influenced gene expression in the vasculature we analysed between group differences using a mixed model two-way ANOVA to site (aortic arch, descending aorta, abdominal aorta, left ventricle and right ventricle) and allocation main effects and their interaction. An overall 5% significance level was preserved for prespecified analysis of between group differences at each site by calculating an FDR protected *p*-value.

Lastly, we investigated whether anatomical heterogeneity existed in gene expression. For these analyses the descending aorta was used as the referent for consistency given there were no between group differences for any gene at this site and were performed as a mixed model two-way ANOVA with site (aortic arch, abdominal aorta, left ventricle and right ventricle) and allocation main effects and their interaction. Each anatomical site was compared with the descending aorta referent using Student's *t*-test and the ho=1 function of SAS. An overall 5% significance level was preserved for prespecified analysis of between group differences at each site by calculating an FDR protected *p*-value.

Analyses were performed with SAS (v9.4, SAS Institute Inc, Cary, NC, USA). Prism version 7.00 for Windows, (GraphPad Software, La Jolla California USA, www.graphpad.com) was used. All tests were two-tailed and $p < 0.05$ was considered significant.

4.3. Results

4.3.1. Weight

Mice were weighed weekly for the first 10 weeks and every 4-6 weeks thereafter (Figure 1). Weights were consistently lower in the calcium group throughout the study period and there

was a significant allocation*time interaction ($p = 0.027$). However, *post hoc* testing at individual time points did not show significant between-group differences. Analysis of changes in weight from baseline also showed a significant allocation*time interaction ($p = 0.029$).

4.3.2. Jelly consumption

From week 6 through to study completion at week 32 we measured the percentage jelly consumption of each mouse in both treatment arms by weighing the jelly that remained at the end of each 2-hour jelly feeding period. Over these 26 weeks the overall jelly consumption in the calcium group and the placebo group was 99.7% and 99.1% respectively.

4.3.3. Serum analysis

Mice were fed jellies 2 hours prior to sacrifice. Serum phosphate was significantly lower in the calcium group (1.61 mmol/L vs 2.28 mmol/L, $p = 0.0007$) (Table 2). There were no significant between group differences in serum corrected calcium, magnesium or albumin concentration.

4.3.4. Tissue calcium quantification

Aortic calcium deposition was investigated in 12 mice and cardiac calcium in 10 mice from each group. Insufficient specimen quantity reduced the numbers for the aortic specimens to 11 in the calcium supplemented jelly group. Mean aortic calcium content was 30% higher in the supplemented animals, but this difference was not statistically significant ($p = 0.28$). There was no between-group difference in cardiac calcium deposition (Figure 4.2).

4.3.5. Histology

No differences were observed between the two treatment groups in either alizarin uptake or von Kossa staining (representative samples presented in Figures 4.3 - 4.4).

Figure 4.1 From 11 weeks of age adiponectin knockout mice were fed calcium and placebo jellies for 32 weeks and were weighed regularly throughout the study: weekly for the first 10 weeks and then every 4-6 weeks thereafter. The upper graph depicts weights of mice in each treatment group at each time point. A mixed model two-way analysis of variance (ANOVA) was used to detect between-group differences in weight over time. There was no significant between-group difference at any of the 16 time points but the overall allocation by time interaction was significant ($p = 0.027$). Given the difference in weight at baseline we performed a further analysis looking at change in weight from baseline with baseline weight as a covariate. This did not alter the allocation by time interaction ($p = 0.029$) and there were no between group differences at any of the 15 time points. Values are mean (95% confidence interval).

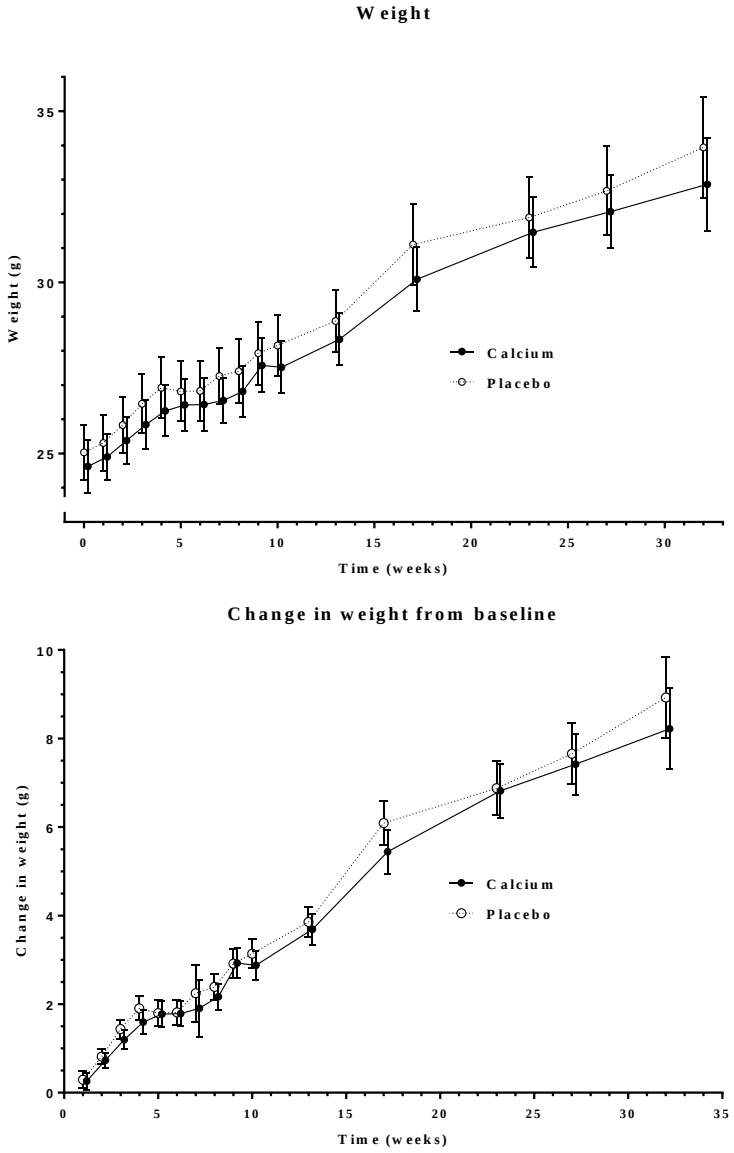


Table 4.1 Serum results for Adiponectin knockout mice that were fed a single daily calcium or placebo jelly five times a week for 32 weeks. Serum sampling was performed at the completion of the study after jelly ingestion. Results are mean (SD).

	Calcium jelly (n=9)	**Placebo jelly (n=9)**	***p*-value**
Calcium (mmol/L)*	2.53 (0.09)	2.51 (0.04)	0.46
Phosphate (mmol/L)	1.61 (0.33)	2.28 (0.31)	0.0007
Magnesium (mmol/L)	0.83 (0.05)	0.79 (0.08)	0.35
Albumin (g/L)	35.3 (1.9)	35.6 (0.9)	0.76

*Corrected total calcium.

4.3.6. Gene expression

In six mice from each group genes implicated in the osteo-chondrogenic transdifferentiation of vascular smooth muscle cells as well in local inhibitors of mineralization, calcium transport and matrix degradation were analysed to elucidate whether calcium supplementation might cause a change in expression. The aorta was analysed in three anatomical parts: aortic arch, descending aorta and abdominal aorta as well as a cardiac sample from each of the left and right ventricle. For *Alpl*, *Bglap*, *ColIa1* and *Ibsp* gene analysis was performed only on the aortic arch and abdominal aorta.

For *Alpl* (Figure 4.5), *Ibsp* (Figure 4.6), *Bglap* (Figure 4.7) and *Scl20a1* (Figure 4.8) gene expression there were no between-group differences at any of the sites analysed. Small but statistically significant between group differences were observed and persisted after adjustment for multiple testing in the expression of *Mmp2* (Figure 4.9) and *Mgp* (Figure 4.10), but were lost in *Anxa6* (Figure 4.11), *Runx2* (Figure 4.12) and *Enpp1* (Figure 4.13). There was a large and significant increase in *ColIa1* (Figure 4.14) expression in the aortic arch of calcium jelly-fed mice.

We investigated whether gene expression differed throughout the aorta and the myocardium using the descending aorta as the referent. There was evidence for heterogeneity in expression for all genes analysed (Figures 4.15 - 4.20) with evidence for differential expression within the aorta (*Runx2* Figure 4.15, *Scl20a1* Figure 4.16, *Mmp2* Figure 4.17). There was also significant allocation*site interaction for *Scl20A1* (Figure 4.16), *Enpp1* (Figure 4.18) and *Mgp* (Figure 4.19). *Mgp* expression in the RV of the calcium supplemented jelly group was 7-12x higher than that in the aorta, whereas this was only 4-5x higher in the placebo group. For *Enpp1,* gene expression was up to 2x higher in the aorta than the DA referent and as low as 0.5x in the heart. *Anxa6* expression (Figure 4.20) in the heart was consistently 0.5x less than that of the aorta in both treatment groups.

Figure 4.2 Deposition of calcium in aortas and hearts of adiponectin knockout mice that were fed calcium supplemented jelly or placebo jelly 5 days per week for 32 weeks. Calcium deposition was measured using an inductively coupled plasma mass spectrometer and normalized to cellular protein content. *p*-values are the result of between group analyses using a mixed model two-way analysis of variance (ANOVA).

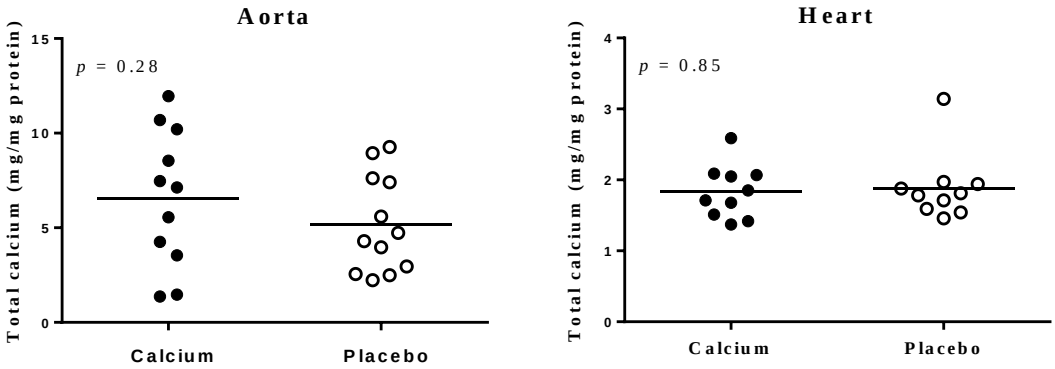


Figure 4.3 Histological specimens underwent Haematoxylin and eosin staining. All images were captured at 40x magnification. **a-c** aortic arch, descending aorta and abdominal aorta of calcium-supplemented group. **d-f** aortic arch, descending aorta and abdominal aorta of placebo group.

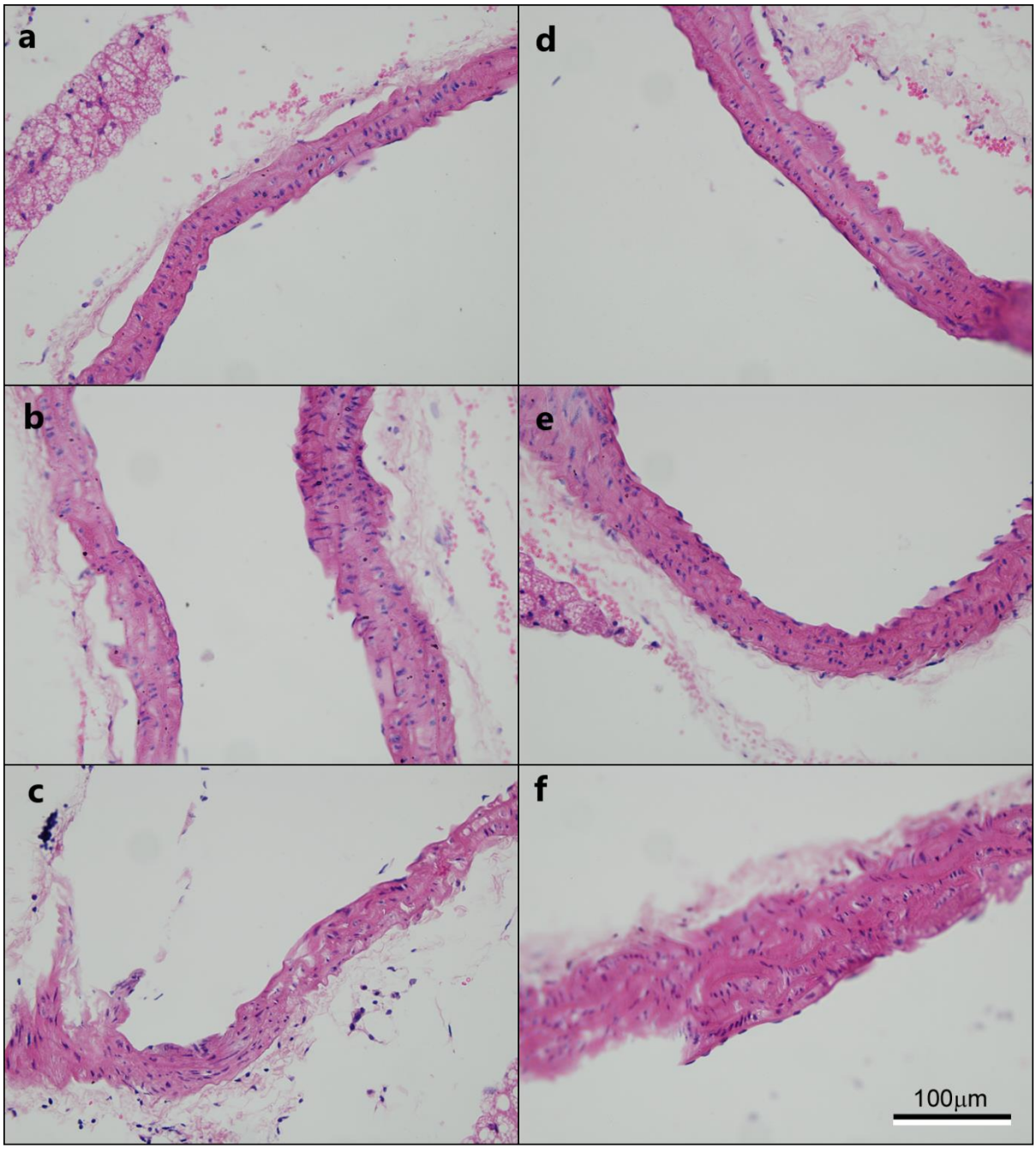


Figure 4.4 Histological specimens underwent von Kossa staining and Alizarin Red S staining. Images were captured at 20x magnification. **a)** Calcium supplemented group, abdominal aorta, von Kossa stain. **b)** Calcium supplemented group, descending aorta, alizarin red s stain. **c)** Placebo group, descending aorta, von Kossa stain. **d)** Placebo group, descending aorta, alizarin red s stain. No specimens stained positively for calcium using either alizarin red s or von Kossa stains.

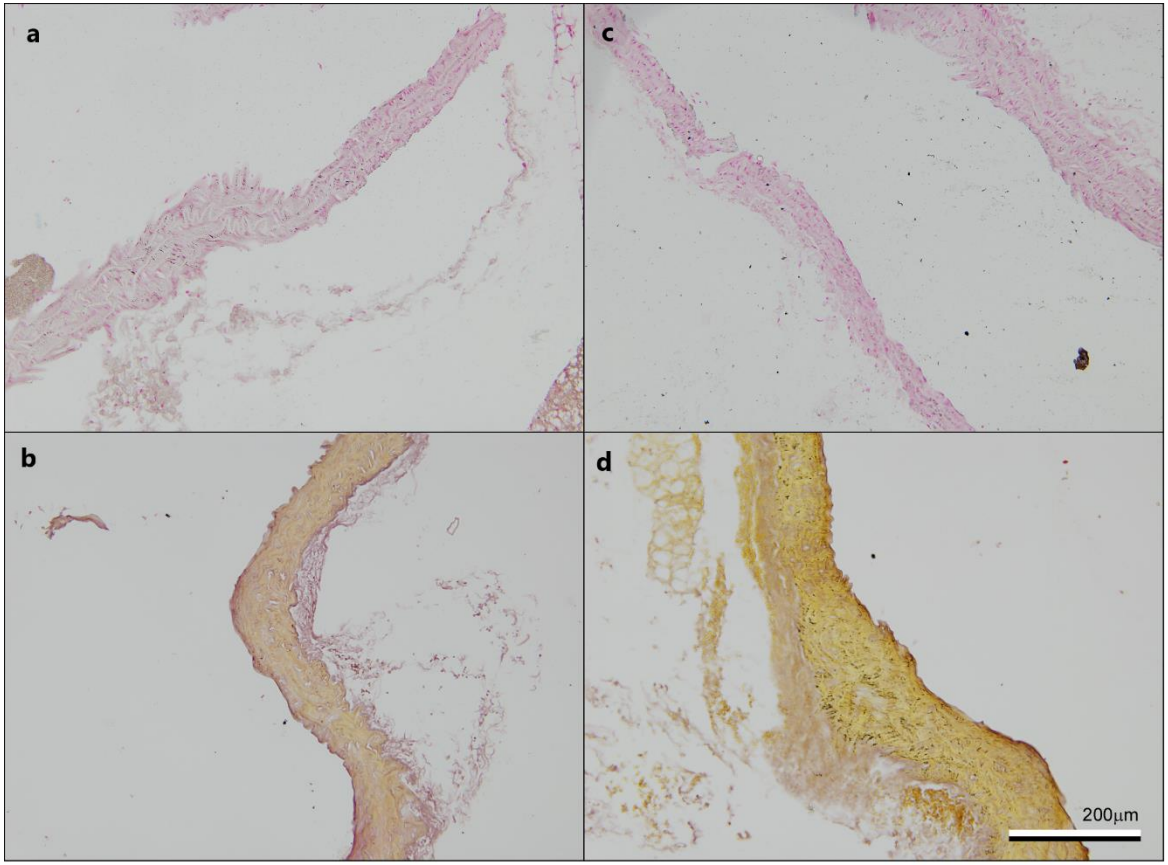


Figure 4.5 Effect of calcium supplemented jelly versus placebo jelly on Alkaline phosphatase (*Alpl)* gene expression in adiponectin knockout mice (*n* = 6). *p*-values are the result of between group analyses using a mixed model two-way analysis of variance (ANOVA) with adjustment for multiple comparisons testing (False Discovery Rate).

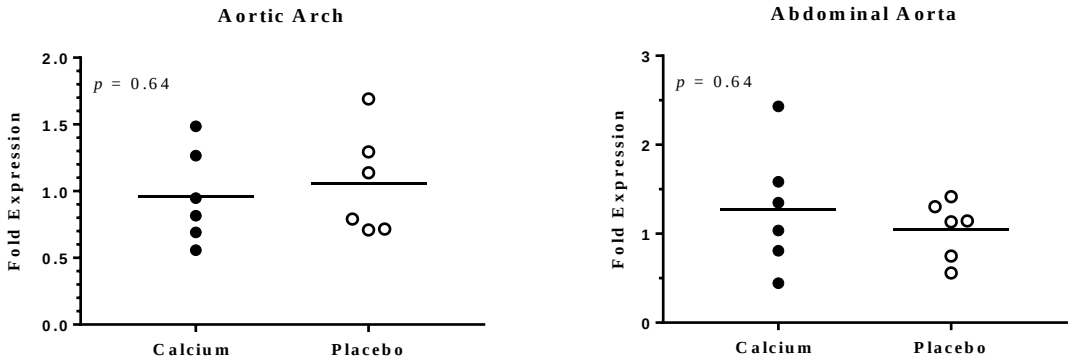


Figure 4.6 Effect of calcium supplemented jelly versus placebo jelly on Bone sialoprotein (*Ibsp*) gene expression in adiponectin knockout mice (*n* = 6). *p*-values are the result of between group analyses using a mixed model two-way analysis of variance (ANOVA) with adjustment for multiple comparisons testing (False Discovery Rate).

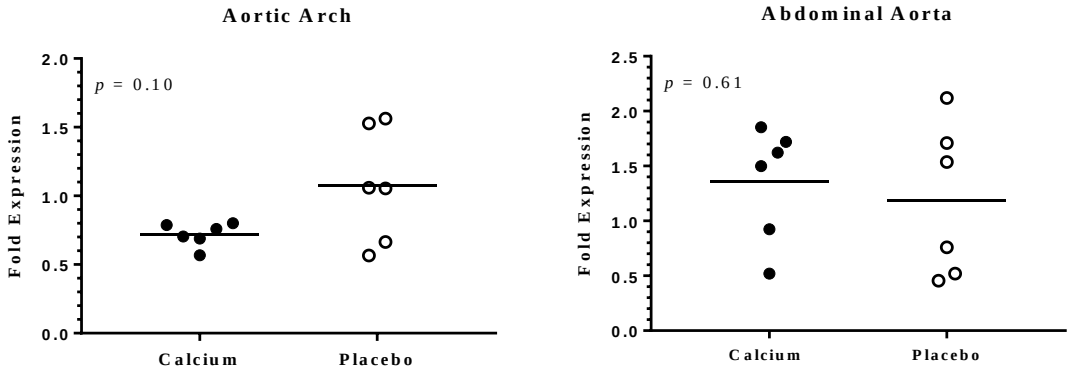


Figure 4.7 Effect of calcium supplemented jelly versus placebo jelly on Bone Gla protein (*Bglap*) gene expression in adiponectin knockout mice (*n* = 6). *p*-values are the result of between group analyses using a mixed model two-way analysis of variance (ANOVA) with adjustment for multiple comparisons testing (False Discovery Rate).

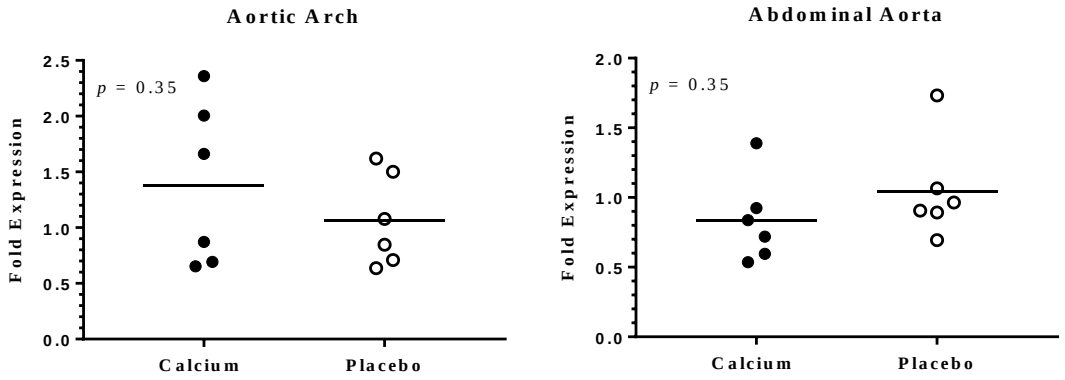


Figure 4.8 Effect of calcium supplemented jelly versus placebo jelly on Sodium-dependent phosphate transporter 1 (*Scl20a1*) gene expression in adiponectin knockout mice (n = 6). p-values are the result of between group analyses using a mixed model two-way analysis of variance (ANOVA) with adjustment for multiple comparisons testing (False Discovery Rate).

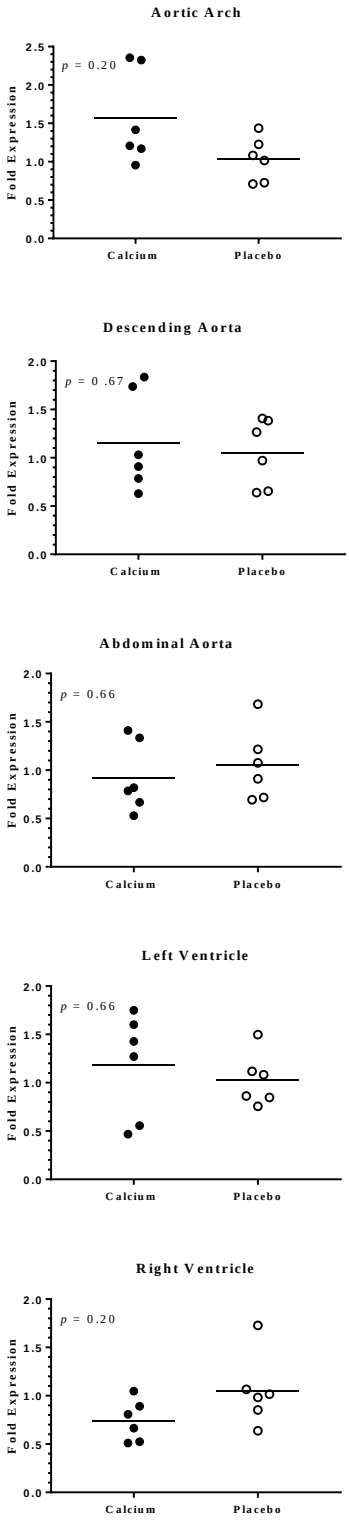


Figure 4.9 Effect of calcium supplemented jelly versus placebo jelly on Matrix metalloprotein 2 (*Mmp2*) gene expression in adiponectin knockout mice (n = 6). p-values are the result of between group analyses using a mixed model two-way analysis of variance (ANOVA) with adjustment for multiple comparisons testing (False Discovery Rate).

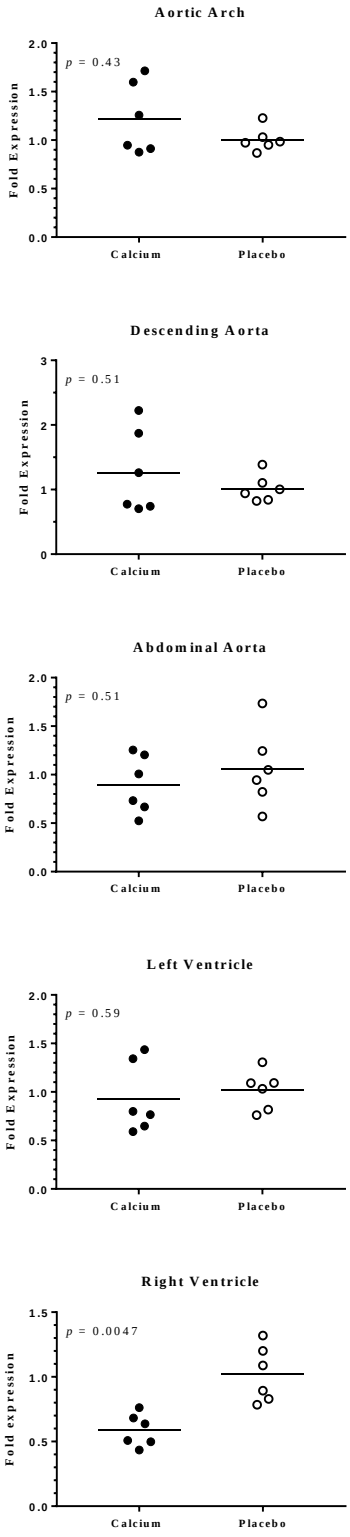


Figure 4.10 Effect of calcium supplemented jelly versus placebo jelly on Matrix gla protein (*Mgp*) gene expression in adiponectin knockout mice ($n = 6$). *p*-values are the result of between group analyses using a mixed model two-way analysis of variance (ANOVA) with adjustment for multiple comparisons testing (False Discovery Rate).

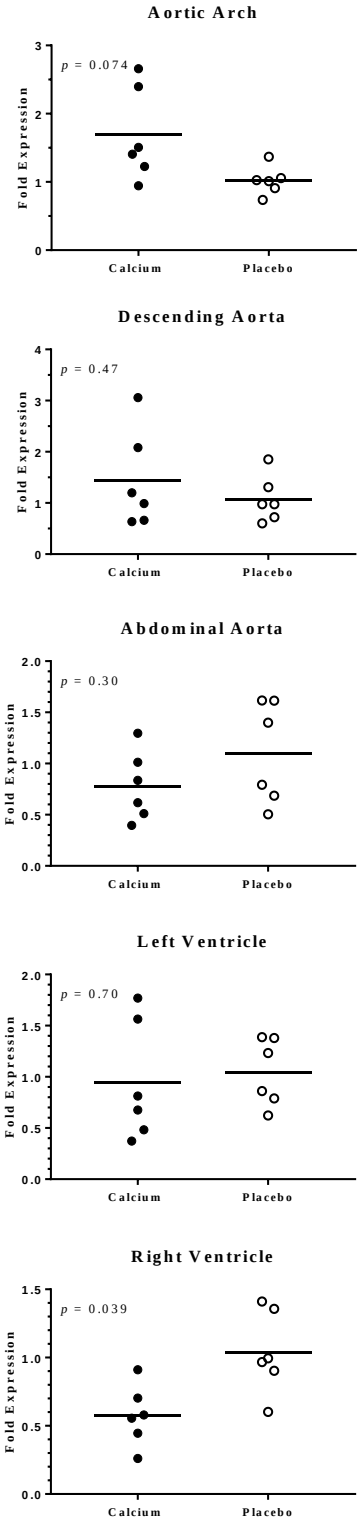


Figure 4.11 Effect of calcium supplemented jelly versus placebo jelly on Annexin A6 (*Anxa6*) gene expression in adiponectin knockout mice (n = 6). p-values are the result of between group analyses using a mixed model two-way analysis of variance (ANOVA) with adjustment for multiple comparisons testing (False Discovery Rate).

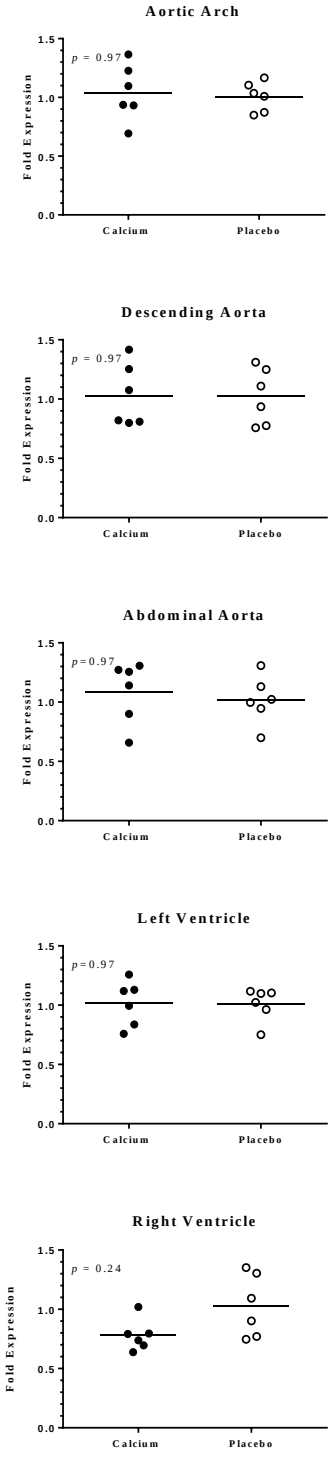


Figure 4.12 Effect of calcium supplemented jelly versus placebo jelly on *Runx2* gene expression in adiponectin knockout mice (n = 6). p-values are the result of between group analyses using a mixed model two-way analysis of variance (ANOVA) with adjustment for multiple comparisons testing (False Discovery Rate).

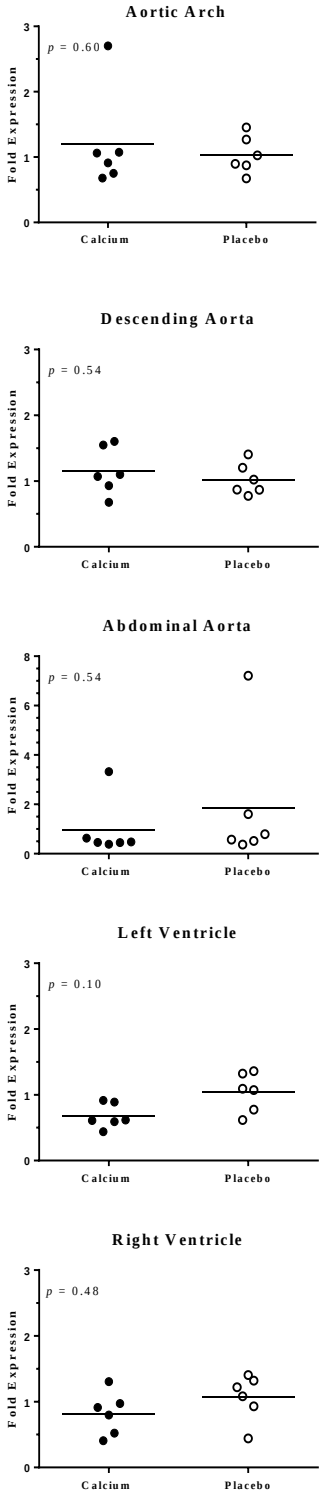


Figure 4.13 Effect of calcium supplemented jelly versus placebo jelly on Ectonucleotide pyrophosphatase/phosphodiesterase 1 (*Enpp1*) gene expression in adiponectin knockout mice (n = 6). p-values are the result of between group analyses using a mixed model two-way analysis of variance (ANOVA) with adjustment for multiple comparisons testing (False Discovery Rate).

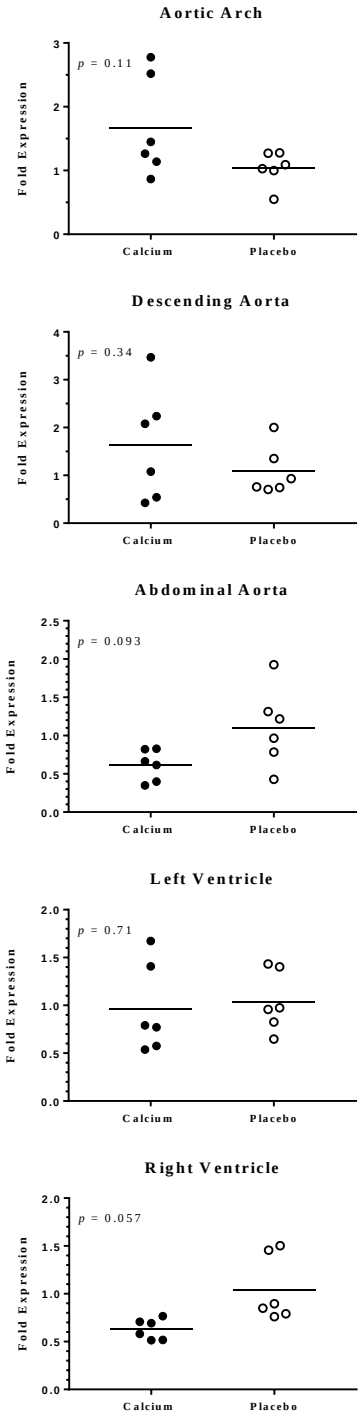


Figure 4.14 Effect of calcium supplemented jelly versus placebo jelly on Collagen, type I, alpha 1 *(ColIa1*) gene expression in adiponectin knockout mice (*n* = 6). *p*-values are the result of between group analyses using a mixed model two-way analysis of variance (ANOVA) with adjustment for multiple comparisons testing (False Discovery Rate).

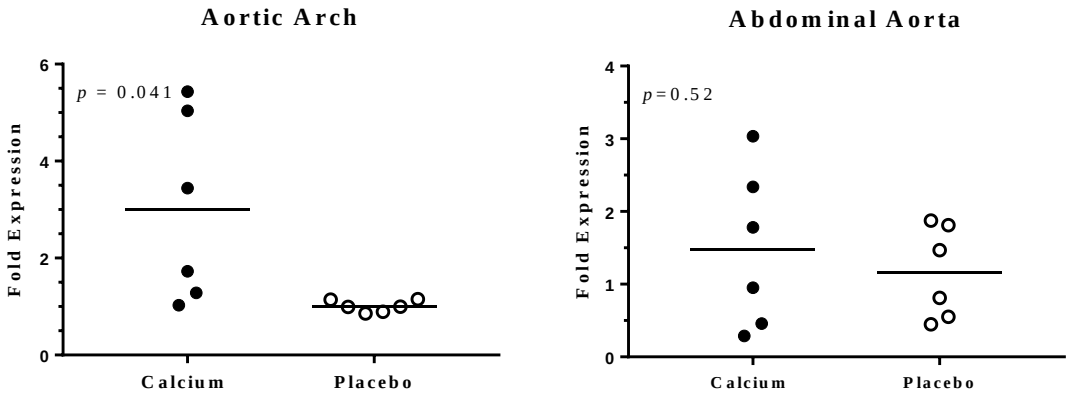
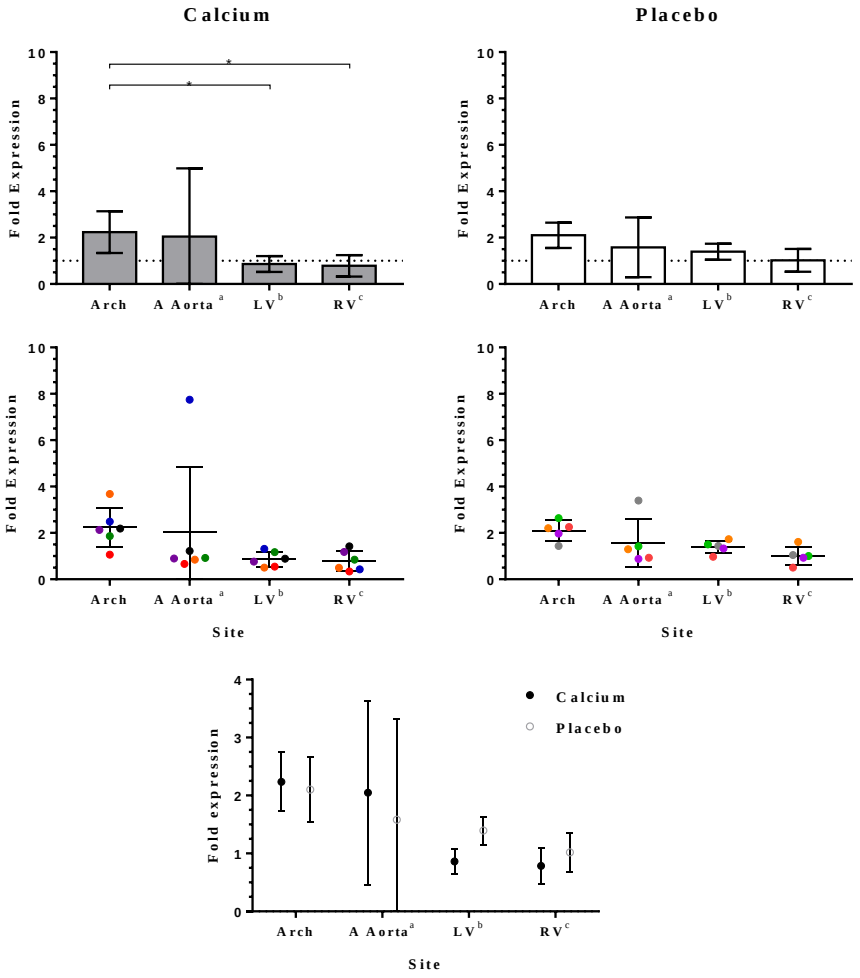


Figure 4.15 Result of calcium supplemented jelly versus placebo jelly on *Runx2* gene expression in adiponectin knockout mice (calcium $n = 6$, placebo $n = 5$). Using the descending aorta as a referent anatomical sites within each treatment group were analysed using a mixed model two-way analysis of variance (ANOVA). Each colour represents a single mouse. Site variation in gene expression was not observed in the placebo group however in mice treated with a calcium jelly *Runx2* gene expression was significantly higher in the aortic arch when compared with the left ventricle ($p = 0.015$) and right ventricle ($p = 0.016$). Allocation by site interaction $p = 0.069$. We adjusted for multiple testing comparisons (False Discovery Rate). There were no significant between-group differences at each of the 4 sites. Results are presented as mean, 95% confidence interval.

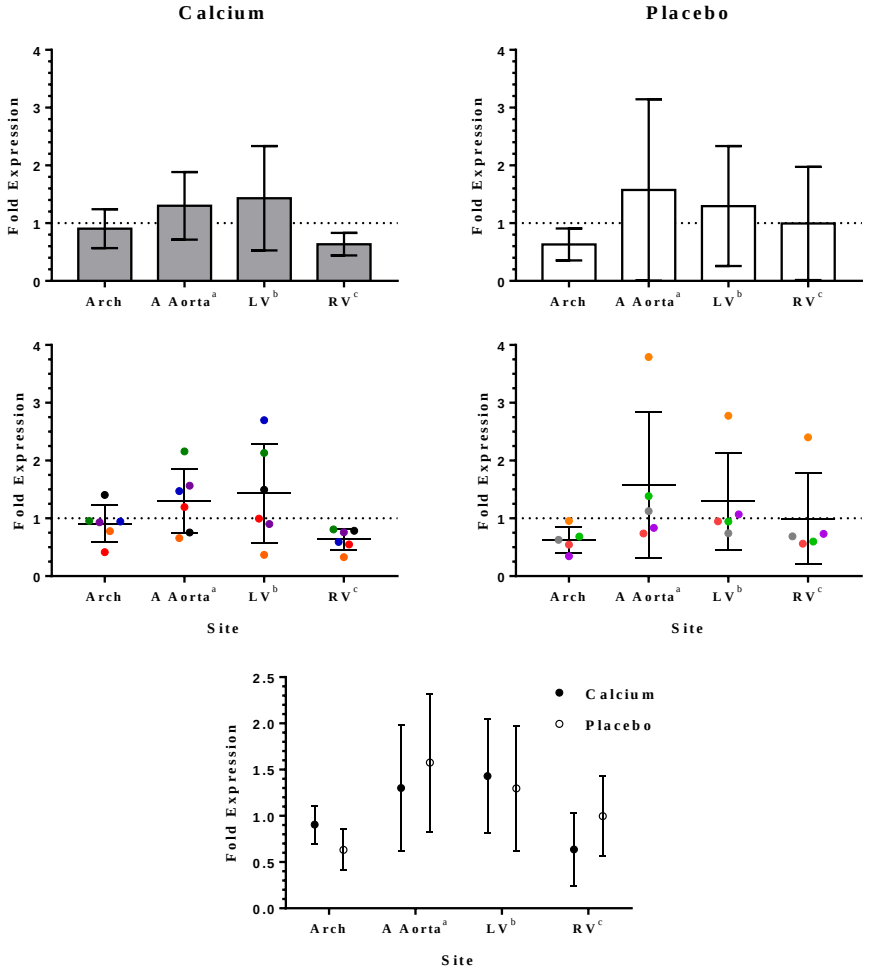


[a] Abdominal Aorta

[b] Left Ventricle

[c] Right Ventricle

Figure 4.16 Result of calcium supplemented jelly versus placebo jelly on Sodium-dependent phosphate transporter 1 (*Scl20a1*) gene expression in adiponectin knockout mice (calcium n = 6, placebo n = 5). Using the descending aorta as a referent anatomical sites within each treatment group were analysed using a mixed model two-way analysis of variance (ANOVA). Each colour represents a single mouse. In the calcium supplemented group gene expression differed between the descending aorta and right ventricle, and in the placebo group between the aortic arch and descending aorta. Site variation in gene expression was not observed in either treatment group. Overall allocation by site interaction p = 0.041. We adjusted for multiple testing comparisons (False Discovery Rate). There were no significant between-group differences at each of the 4 sites. Results are presented as mean, 95% confidence interval.

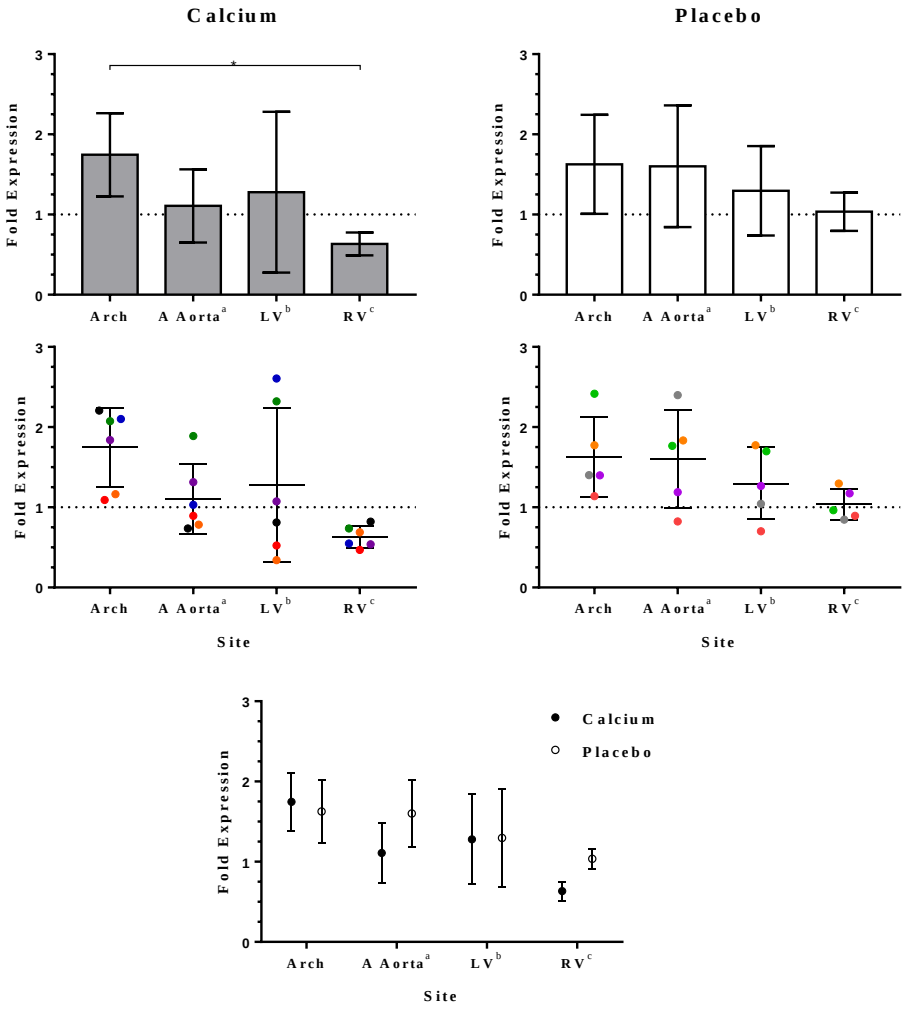


[a] Abdominal Aorta

[b] Left Ventricle

[c] Right Ventricle

Figure 4.17 Result of calcium supplemented jelly versus placebo jelly on matrix metalloprotein 2 (*Mmp2*) gene expression in adiponectin knockout mice (calcium $n = 6$, placebo $n = 5$). Using the descending aorta as a referent anatomical sites within each treatment group were analysed using a mixed model two-way analysis of variance (ANOVA). Each colour represents a single mouse. Site variation in gene expression was not observed in the placebo group. In mice receiving high calcium chow *Mmp*2 expression was significantly higher in the aortic arch compared with the right ventricle ($p = 0.002$). Allocation by site interaction $p = 0.21$. We adjusted for multiple comparisons testing (False Discovery Rate). There was a significant between-group difference at the right ventricle $p = 0.0061$. Results are presented as mean, 95% confidence interval.

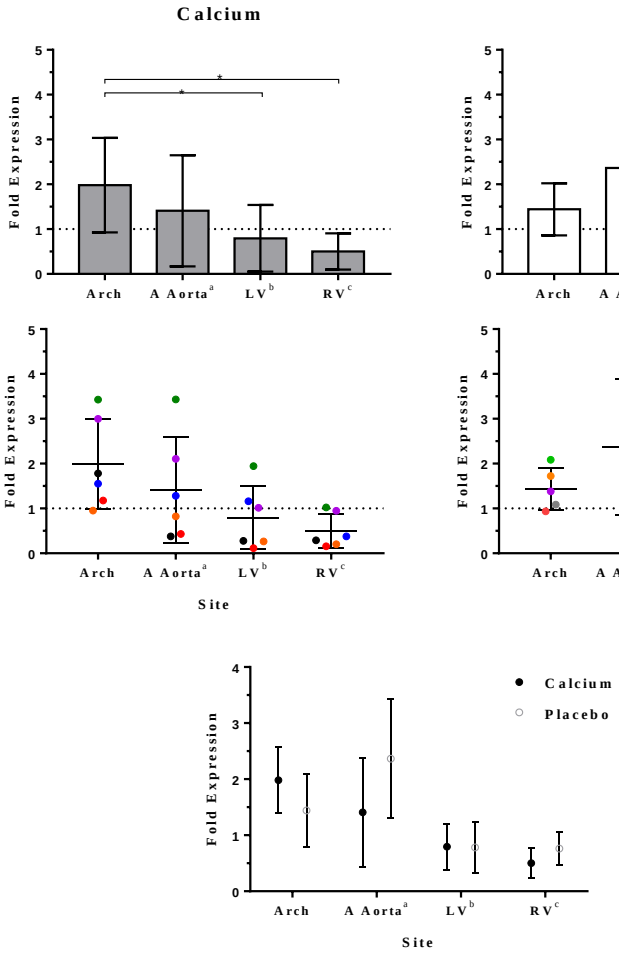


[a] Abdominal Aorta

[b] Left Ventricle

[c] Right Ventricle

Figure 4.18 Result of calcium supplemented jelly versus placebo jelly on Ectonucleotide pyrophosphatase/phosphodiesterase 1 (*Enpp1*) gene expression in adiponectin knockout mice (n = 6). Using the descending aorta as a referent anatomical sites within each treatment group were analysed using a mixed model two-way analysis of variance (ANOVA). Each colour represents a single mouse. In mice treated with placebo jelly, *Enpp1* gene expression did not significantly vary across anatomical sites. However, calcium jelly treatment resulted in an increase *Enpp1* expression in the aortic arch compared with the left ventricle (p = 0.0011) and right ventricle (p = 0.0006). Allocation by site interaction p = 0.028. We adjusted for multiple comparisons testing (False Discover Rate). There were no significant between-group differences at each of the 4 sites. Results are presented as mean, 95% confidence interval.

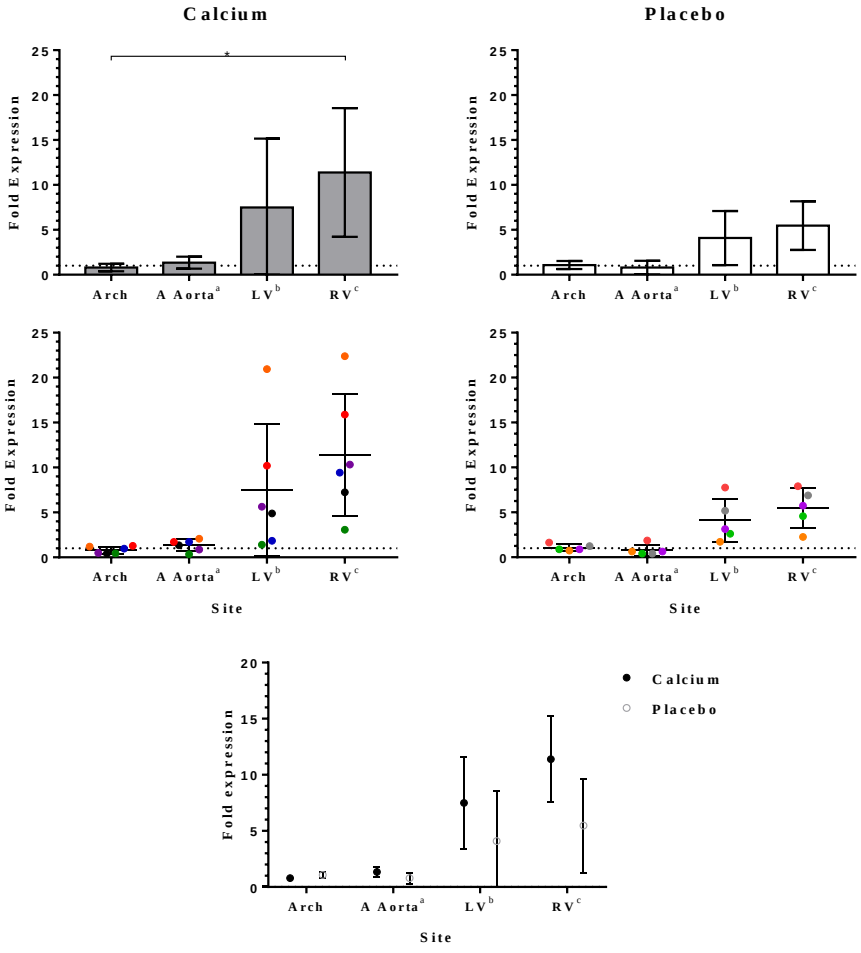


[a] Abdominal Aorta

[b] Left Ventricle

[c] Right Ventricle

Figure 4.19 Result of calcium supplemented jelly versus placebo jelly on Matrix gla protein (*Mgp*) gene expression in adiponectin knockout mice (calcium $n = 6$, placebo $n = 5$). Using the descending aorta as a referent anatomical sites within each treatment group were analysed using a mixed model two-way analysis of variance (ANOVA). Each colour represents a single mouse. In mice fed a placebo jelly there was no significant difference in *Mgp* expression throughout the sites analysed. However, in mice fed a calcium jelly *Mgp* expression resulted in a significant increase in expression right ventricle compared with the aortic arch ($p = 0.0043$). Allocation by site interaction $p = 0.0071$. We adjusted for multiple comparisons testing (False Discover Rate). There were no significant between-group differences at each of the 4 sites. Results are presented as mean, 95% confidence interval.

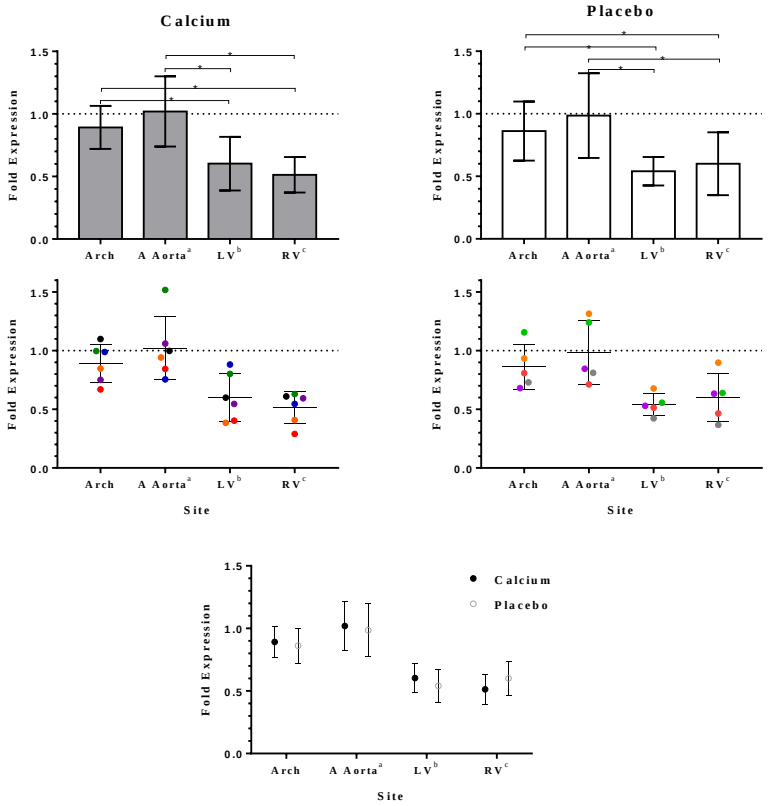


[a] Abdominal Aorta

[b] Left Ventricle

[c] Right Ventricle

Figure 4.20 Result of calcium supplemented jelly versus placebo jelly on Annexin A6 (*Anxa6*) gene expression in adiponectin knockout mice (calcium $n = 6$, placebo $n = 5$). Using the descending aorta as a referent anatomical sites within each treatment group were analysed using a mixed model two-way analysis of variance (ANOVA). Each colour represents a single mouse. In the placebo group *Anxa6* expression was significantly higher in the aortic arch compared with the left ventricle ($p = 0.012$) and right ventricle ($p = 0.047$), and higher in the abdominal aorta compared with the left ventricle ($p = 0.027$) and right ventricle ($p = 0.014$). These differences were preserved with calcium jelly ingestion with gene expression significantly higher in the aortic arch compared with the left ventricle ($p = 0.0132$) and right ventricle ($p = 0.0025$), and higher in the abdominal aorta compared with the left ventricle ($p = 0.0232$) and right ventricle ($p = 0.0011$). Allocation by site interaction $p = 0.18$. We adjusted for multiple comparisons testing (False Discover Rate). There were no significant between-group differences at each of the 4 sites. Results are presented as mean, 95% confidence interval.



[a] Abdominal Aorta

[b] Left Ventricle

[c] Right Ventricle

4.4. Discussion

In this study we investigated whether 120 mg of elemental calcium fed as a single daily jelly, resulting in a daily calcium intake of 3x normal, would change vascular calcium deposition and gene markers for calcification in adiponectin knockout mice. We found significant between-group differences in weight over time, serum phosphate level, and gene expression. No differences were observed in tissue calcium deposition or histology.

In this study, mice receiving calcium jelly gained less weight than the placebo group. This is consistent with many animal and human studies (682-685). Although the literature is not unanimous (686-688) there is substantial evidence for calcium attenuating weight gain on a high fat diet as well as increasing weight loss in calorie restricted diets. Dairy calcium appears to be more beneficial than calcium supplements, with an analysis of milk proteins identifying lactoferrin as having the greatest impact on weight loss (685). Some studies and a subsequent meta-analysis (689) have failed to find a treatment effect but this may be secondary to study flaws and the lack of homogeneity in the studies compared (690). The mechanisms underpinning the effect calcium has on body fat and weight are likely to be multifactorial. Dietary calcium reduces intra-adipocyte calcium concentrations through the downregulation of circulating PTH and vitamin D, the latter of which may also cause thermogenic uncoupling reducing metabolic efficiency (683,691).

Increases in intra-adipocyte calcium lead to lipogenesis with reduced calcium concentrations resulting in lipolysis. Oral calcium also appears to improve gut health in a prebiotic manner by improving gut microbiota with a reduction in the harmful species endotoxin LPS and chlamydia, and increasing beneficial microbes of the *Bacteroidetes* and *Actinobacteria* species (692). Calcium also decreases gut fat absorption by sequestration of fatty acids and can decrease weight through increasing satiety. The calcium sensing receptor may also play

an important role in adipocyte-based mechanisms providing an important link between calcium, vitamin D and the inflammatory sequalae of obesity (690). Overall, these beneficial effects of oral calcium result in an improvement in metabolic status.

The fate of serum phosphate is well documented following an intravenous (IV) infusion of calcium but the data are sparse on the effect after an oral calcium load. Although intravenously administered phosphate reduces serum calcium, intravenously administered calcium does not reduce serum phosphate. In animals and humans the administration of IV calcium results in an elevation of serum phosphate and a phosphate antidiuresis (693-699). This is likely a PTH effect with increased serum calcium concentrations reducing circulating PTH and therefore, in the absence of PTH-induced degradation of the proximal tubule phosphate cotransporters NaPi-IIa and NaP-IIc, a permissive renal reabsorption of phosphate ensues (700). The phosphatonins, the best known of which is FGF-23, also affect renal phosphate handling in this way but their actions are delayed in onset by many hours to days as opposed to PTH which can have its effect in 2 hours (700). Sudden dietary increases in phosphate can lead to internalization of the type IIa Na-Pi contransporter from 4-6 hours post oral ingestion in rats with subsequent phosphate diuresis although this mechanism is unlikely to play a role here (701). Serum phosphate following an oral calcium load behaves differently. Bristow *et al*. administered calcium as a calcium supplement (citrate or carbonate) or microcrystalline hydroxyapatite showing an initial decrease in serum phosphate at 2 hours for both preparations, followed by an elevation above baseline in those taking microcrystalline hydroxyapatite at 4 hours and normalizing at 6 hours (326). The control group also showed a decrease in serum phosphate at 2 hours. Narva *et al.* also report a decrease in phosphate both in treatment and control groups 2 hours following milk ingestion in postmenopausal women (702). Here the treatment group received fermented milk with the control group ingesting unfermented milk. Kärkkäinen *et al*. also performed serial serum

testing for calcium and phosphate following the ingestion of four calcium-rich foods, once again showing an increase in calcium and an initial decrease in phosphate before levels returned to either baseline or elevated above baseline depending on the treatment arm. Here the control group did not receive calcium and the observed serum phosphate dip was identical to those in the treatment groups (703). In these three studies interventions were carried out after an overnight fast and were accompanied by breakfast. The induced hypophosphatemia observed in these studies is therefore likely to be an insulin effect as insulin and therefore glucose is known to produce decreases in serum phosphate (704,705). In our study, treatment and placebo groups received identical chow and were not fasted overnight prior to intervention. This could explain the lack of perturbation in serum phosphate in the placebo group although we have not measured phosphate before and after feeding. The jelly formulation was identical for both, apart from the addition of calcium, therefore it would appear that the reduction in serum phosphate seen in those mice receiving a calcium jelly is a true calcium effect. Given the presence of the calcium sensing receptor in the intestinal tract and its ability to modulate colonic activity, it is plausible that agonist activity in the intestinal tract could reach other organ systems. As serial serum measures were not obtained, we do not know if the reduction in serum phosphate was followed by a subsequent elevation as observed by Bristow *et al*., although this phenomenon was not consistently demonstrated in the calcium-treated arms of Kärkkäinen *et al*. In the aforementioned studies, oral calcium resulted in an increase in serum calcium which we did not observe although our study was of a much longer duration and therefore limits comparability. As the calcium effect on sera has not been documented in mice using repeated measures over time, we do not know the point at which serum calcium peaks and therefore we may have missed this peak at the time of our sampling. Alternatively, the oral calcium jelly did not affect serum calcium.

In this study calcium treatment resulted in a 30% increase in aortic calcium as measured by inductively-couple plasma spectroscopy although this was not statistically significant and no calcification was observed on histological specimens. This is in stark contrast to the results of Luo *et al*. who found that 6-week-old male adiponectin knockout mice fed normal chow for 30 weeks had a significant increase in calcium deposition within the descending aorta compared with their counterparts who were 10 or 20 weeks younger (677). It is difficult to understand why our study differs. The explanation perhaps lies within adiponectin knockout and epigenetic modifications influencing phenotypic penetration as we were unable to reproduce the vascular phenotype in our placebo treated animals described by Luo *et al*. A further possible explanation could be from unidentified differences in the way the animals were managed (648).

We found small but significant between-group differences in the expression of *Mmp2* and *Mgp*, the biological significance of which is uncertain. However, the 3-fold increase in expression of *ColIa1* in the aortic arch of the calcium fed group compared with the placebo group is more compelling. Type I collagen is the most abundant type of collagen and is expressed in almost all connective tissues. In bone the collagen matrix contributes mainly to bone toughness, that is its ability to absorb energy. In blood vessels, type 1 collagen is produced by endothelial cells and vascular smooth muscle cells where its role is to provide tensile strength and mechanical integrity and as they are not elastically deformable, setting the limits by which the blood vessel can expand (429). The loss of this important function is manifested in aneurysmal disease where this fibril forming collagen is reduced or degraded by matrix metalloproteinases. Accordingly, mice with a genetic defect in functioning Type I collagen die in utero because of vessel rupture (706). Collagen I is therefore important in normal vessel function but also plays a core role in vessel pathology. In atherosclerosis the thickened intima is 1/3 collagen I increasing to 76% in the fibrous plaque. Here collagen,

which in this setting may also be synthesized by cells of an osteoblastic/chondrocytic phenotype, is important for plaque stability with increased collagen degradation leading to plaque instability and vulnerability to rupture. Following vascular injury collagen plays an important role in facilitating vascular smooth muscle cell switch to a secretory phenotype and provides a scaffold for smooth muscle cell migration. However, overproduction can also promote vessel stenosis and its sequalae of angina, and restenosis following percutaneous interventions. As mentioned above, collagen promotes the secretory phenotype of SMC facilitating their migration to sites of injury. In atherosclerosis it also binds LDL in the vessel wall, increases calcification with increased ALP production and calcium incorporation and nodule formation, promotes platelet activation, and induces apoptosis. Stretch also stimulates collagen production perhaps providing vessel stability against aneurysm and rupture. Although we have not measured stretch in our study it would seem unlikely that this is the cause of the increase in *ColIa1* expression. A more likely explanation is that the calcium supplemented jelly led to an increase in vessel injury by any number of possible mechanisms and this in turn led to an increase in collagen expression. Given this difference in expression was only observed in the aortic arch it would point to this anatomical site as being vulnerable, a finding substantiated by Leroux-Berger *et al.* (707). An extension to this is that at anatomical sites already under stress and subject to injury, serum alterations induced by calcium supplemented jelly promoted an increase in *ColIa1* expression. However only 3 of the 6 mice showed elevated *ColIa1* expression and therefore caution should be exercised in interpretation.

As a result of differing embryological origins, different anatomical segments of the aorta are thought to possess smooth muscle cell functional diversity. Previously, many have interpreted the vascular variation in atherosclerosis and calcification as secondary to flow dynamics. In 1964 Haimovici *et al.* demonstrated the predilection of the abdominal aorta to atherosclerosis

irrespective of its anatomical position (708). By placing homografts of the abdominal aorta in the thoracic position and vice versa they demonstrated that mechanical factors are not as important as location of embryological origin in the responsiveness of the aorta to a pro-atherosclerotic milieu with the abdominal aorta showing increased atherosclerosis compared with the thoracic aorta. The adult aorta comprises vascular smooth muscle cells from the transient proepicardium of the lateral plate mesoderm (coronary arteries), splanchnic mesoderm of the lateral plate mesoderm (aortic root), neural crest (ascending aorta and arch as well as arch vessels) and somites (descending aorta) (709). In chick embryos, vascular smooth muscle cells respond differently to a single stimulus in a lineage-dependent fashion with the suggestion that this lineage dependent response may be retained in adults (710). Indeed, this has been verified by the work Pfaltzgraff *et al.* (711). Our findings that the expression of some genes implicated in vascular calcification vary by anatomical location in the vascular tree are in keeping with this also. An interesting finding from this study that deserves further exploration is that *Mgp* expression is increased in the heart relative to the aorta, a difference accentuated with calcium jelly treatment. Matrix gla protein is a known inhibitor of vascular calcification however interestingly Mustonen *et al.* have demonstrated that MGP is upregulated in the presence of cardiac overload before the development of left ventricular hypertrophy (712). If the increased aortic *ColIa1* expression in our mice has led to an increase in vascular stiffness then the subsequent increase in cardiac overload could explain this increase in cardiac *Mgp* expression. Further work is required to understand whether in this setting matrix gla protein is upregulated to prevent calcification or instead a mediator of cardiac hypertrophy.

In this exploratory study we have found some promising leads regarding the effect of calcium on the vasculature that require further investigation. The consequence of an oral calcium supplement on calcium deposition in the aorta and also in *ColIa1* expression in the aortic arch

warrant more rigorous evaluation in an adequately powered study. Additionally, these could also be further investigated in an *in vivo* model with a more striking vascular calcification phenotype.

5. EFFECT OF HIGH CALCIUM DIET ON MOUSE SERUM PROFILE AND GENE EXPRESSION

5.1. Method

For 32 weeks mice were housed in cages with littermates according to treatment allocation of high calcium chow (2.75% Calcium, Teklad rodent diet TD.10948, Harlan TM) or normal chow (1.01% calcium) and water ad libitum. Chow and water were refreshed daily as required and mice were monitored for adverse effects according to local protocol.

Please refer to Chapter 4 for detailed methods.

5.2. Results

5.2.1. Weight

Adiponectin knockout mice randomised to high calcium chow and normal chow were weighed weekly for the first 6 weeks and every 4-6 weeks thereafter (Figure 5.1). There was a significant allocation*time interaction ($p < 0.0001$) in weights over the 32-week study duration, however there were no significant between group differences at any of the 13 time points. We repeated the analysis removing any variation that may have been introduced at baseline by calculating change in weight from baseline. Allocation*time interaction was unchanged. Inspection of the graphs do suggest a striking difference however from week 13 body weight tended to be lower in the high calcium chow group.

5.2.2. Serum analysis

Serum corrected calcium was significantly higher, and serum phosphate was significantly lower in the high calcium chow group compared with mice fed normal chow. There were no between group differences in magnesium and albumin (Table 5.1).

Figure 5.1 From 11 weeks of age adiponectin knockout mice were fed high calcium chow and normal chow for 32 weeks and were weighed regularly throughout the study: weekly for the first 6 weeks and then every 4-6 weeks thereafter. The upper graph depicts weights of mice in each treatment group at each time point. A mixed model two-way analysis of variance (ANOVA) was used to detect between-group differences in weight over time. There was no significant between-group difference at any time point but the overall allocation by time interaction was significant ($p < 0.0001$). Values are mean (95% confidence interval).

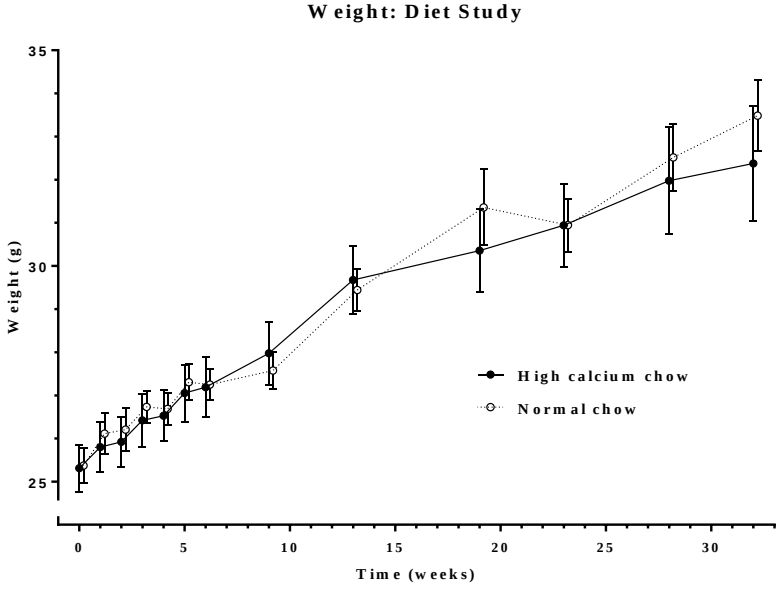


Table 1 Serum results for Adiponectin knockout mice that were fed high calcium chow or normal chow ad libitum for 32 weeks. Serum analysis was performed at study completion. Mice were not fasted prior to serum sampling. Results are mean (SD). Between group differences were analysed using Student's *t*-test.

	High Calcium Chow (n=9)	**Normal Chow (n=7)**	***p*-value**
Calcium*	2.68 (0.21)	2.37 (0.12)	0.0036
Phosphate	1.41 (0.36)	2.07 (0.29)	0.0015
Magnesium	0.83 (0.13)	0.77 (0.11)	0.33
Albumin	36.4 (1.2)	36.6 (1.0)	0.83

*Corrected calcium

5.2.3. Calcium quantification

There were no between group differences in the amount of calcium present in either the heart or aorta (Figure 5.2).

5.2.4. Histology

No differences were observed between the two treatment groups in either alizarin uptake or von Kossa staining (some samples are presented in Figures 5.3 - 5.4).

Figure 5.2 Deposition of calcium in aortas and hearts of adiponectin knockout mice that were fed high calcium chow or normal chow for 32 weeks (n = 11) was measured using an inductively coupled plasma mass spectrometer and normalized to cellular protein content. p-values are the result of between group analyses using a mixed model two-way analysis of variance (ANOVA).

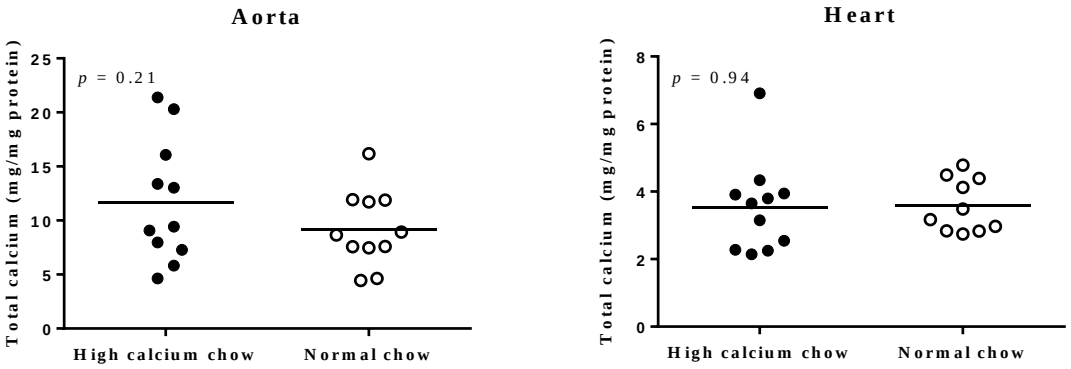


Figure 5.3 Histological specimens underwent haematoxylin and eosin staining. **a-c** images of high calcium chow group; aortic arch, descending aorta and abdominal aorta respectively. Images captured at 40x magnification. **d-e** images captured of full length of aorta from normal chow group. 10x, 20x and 40x respectively.

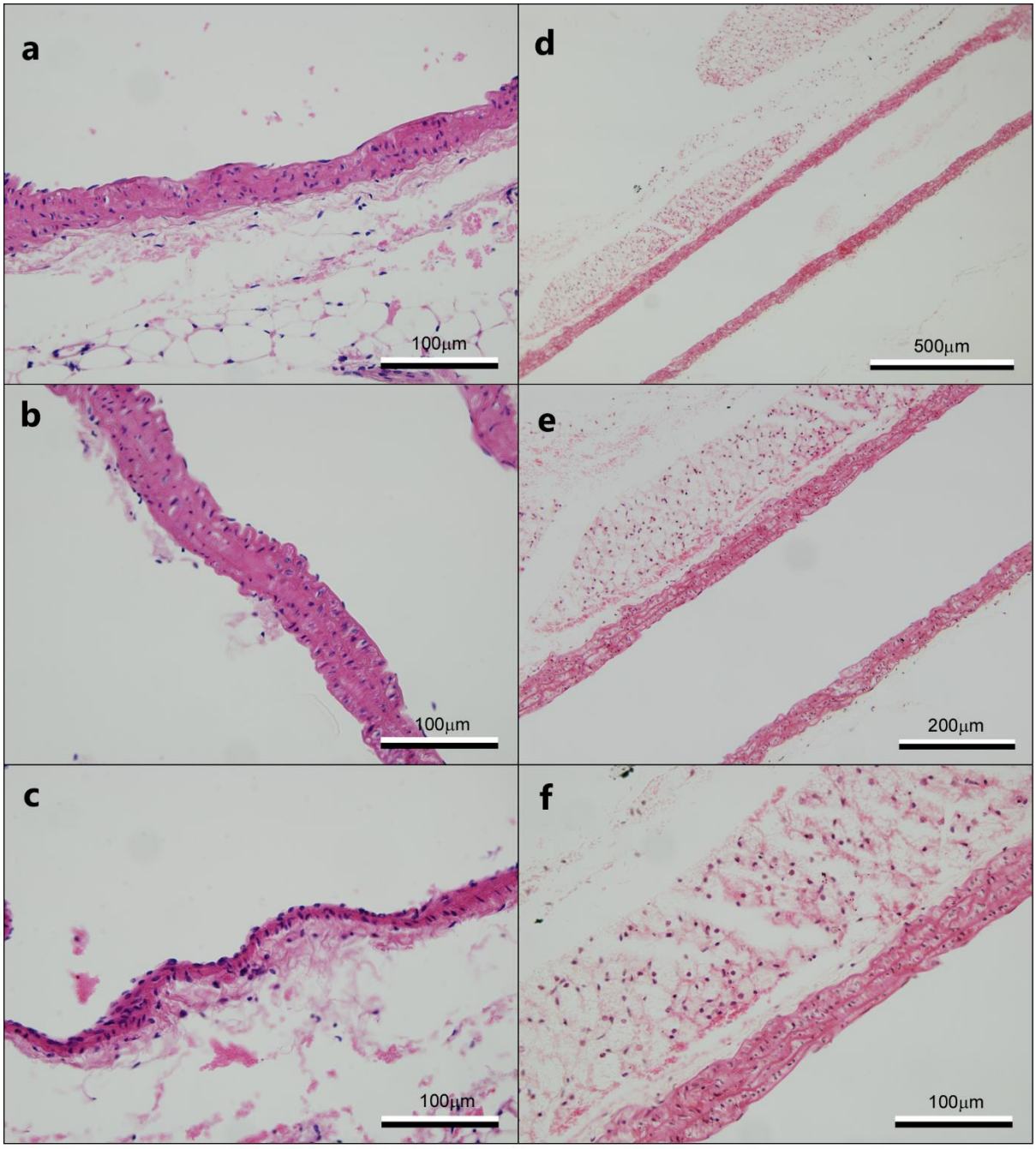
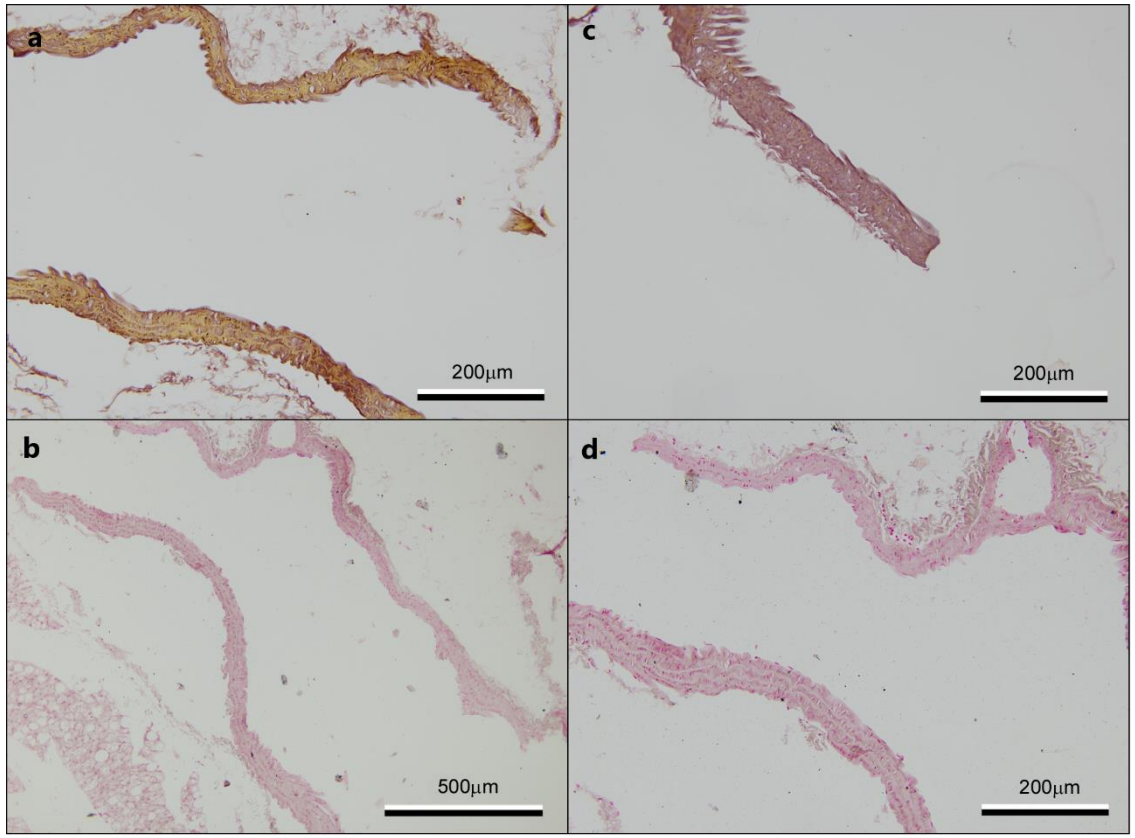


Figure 5.4 Histological specimens underwent von Kossa staining and Alizarin Red S staining. **a)** high calcium chow, descending aorta, alizarin red s stain. Image captured at 20x magnification. **b)** high calcium chow, descending aorta, von Kossa stain. Image captured at 10x magnification. **c)** normal chow, descending aorta, alizarin red s stain. Imaged captured at 20x magnification. **d)** high calcium chow, descending aorta, von Kossa stain. Image captured at 20x magnification. No specimens stained positively for calcium using either alizarin red s or von Kossa stains. No specimens stained positively for calcium using either alizarin red s or von Kossa stains.



5.2.5. Gene expression

In 6 mice from each group genes implicated in the osteo-chondrogenic transdifferentiation of vascular smooth muscle cells as well as local inhibitors, calcium transport and matrix degradation were analysed to elucidate whether calcium supplementation might instigate a change in expression. The aorta was analysed in three anatomical parts: aortic arch (including the ascending aorta), descending aorta and abdominal aorta as well as a cardiac sample from each of the left and right ventricle. For *Alp*, *Bglap*, *ColIa1* and *Ibsp*, gene analysis was performed only on the aortic arch and abdominal aorta as these sites have a propensity for calcification in the human aorta. The RNA quality for an aortic arch specimen of each group was compromised during processing leaving five specimens for analysis.

High calcium chow resulted in a ~25% decrease in Bglap (Figure 5.5) expression in the aortic arch compared with normal chow. No between-group differences were observed in any of the remaining genes analysed (Figures 5.6 – 5.14).

We analysed gene expression across differing anatomical segments of the aorta and heart using the descending aorta as the referent. Anatomical heterogeneity in gene expression was observed in all genes (Figures 5.15 – 5.20) with variation present within the aorta (*Mmp2*, *Mgp* and *Enpp1*). *Mgp* expression was increased by 5x in the heart relative to the aorta and descending aorta in both treatment groups (Figure 5.20). Table 5.2 and 5.3 present summary data for both the calcium supplement study and the diet study. Table 5.2 presents a summary gene analysis results and Table 5.3 depicts whether each anatomical segment differed from the referent.

Figure 5.5 Effect of high calcium chow versus normal chow on bone Gla protein (*Bglap*) gene expression in adiponectin knockout mice (aortic arch $n = 5$, abdominal aorta $n = 6$). *p*-values are the result of between group analyses using a mixed model two-way analysis of variance (ANOVA), adjusted for multiple comparisons testing (False Discovery Rate).

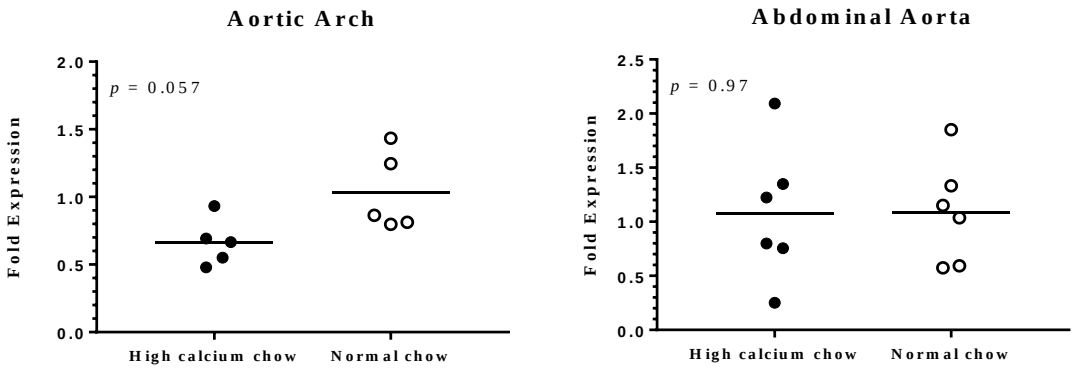


Figure 5.6 Effect of high calcium chow versus normal chow on alkaline phosphatase (*Alp*) gene expression in adiponectin knockout mice (aortic arch $n = 5$, abdominal aorta $n = 6$). *p*-values are the result of between group analyses using a mixed model two-way analysis of variance (ANOVA), adjusted for multiple comparisons testing (False Discovery Rate).

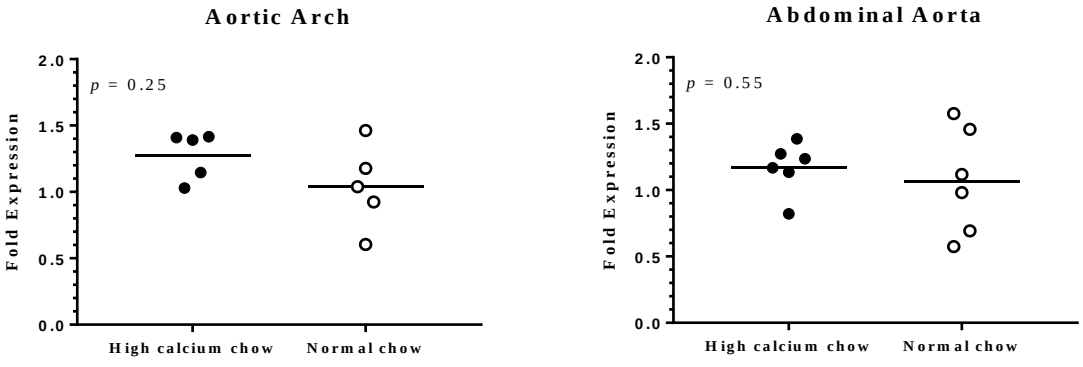


Figure 5.7 Effect of high calcium chow versus normal chow on collagen, type I, alpha 1 (*ColIa1*) gene expression in adiponectin knockout mice (aortic arch *n* = 5, abdominal aorta *n* = 6). *p*-values are the result of between group analyses using a mixed model two-way analysis of variance (ANOVA), adjusted for multiple comparisons testing (False Discovery Rate).

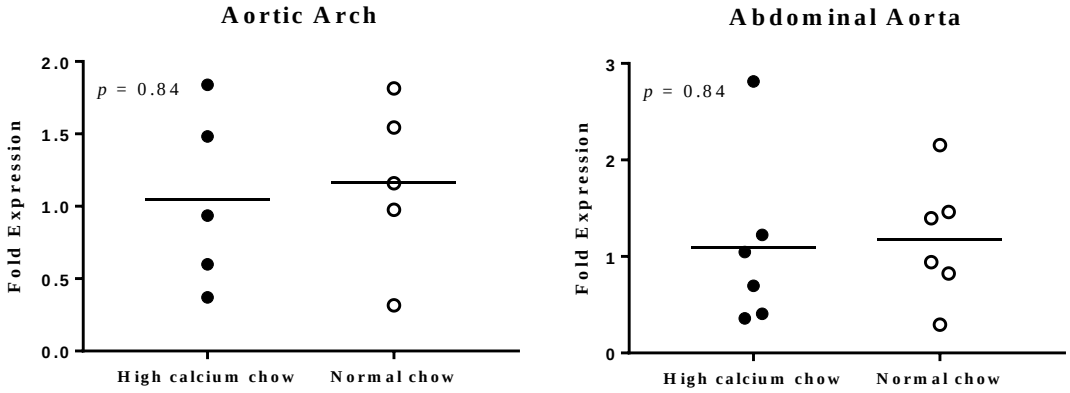


Figure 5.8 Effect of high calcium chow versus normal chow on bone sialoprotein (*Ibsp*) gene expression in adiponectin knockout mice (aortic arch *n* = 5, abdominal aorta *n* = 6). *p*-values are the result of between group analyses using a mixed model two-way analysis of variance (ANOVA), adjusted for multiple comparisons testing (False Discovery Rate).

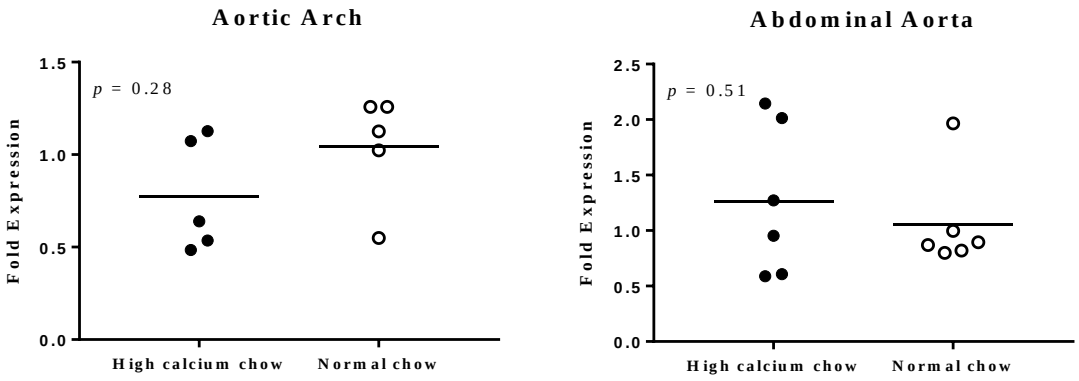


Figure 5.9 Results of *Runx2* gene expression between adiponectin knockout mice taking high calcium chow and normal chow (n = 6, aortic arch n = 5). p-values are the result of between group analyses using a mixed model two-way analysis of variance (ANOVA), adjusted for multiple comparisons testing (False Discovery Rate).

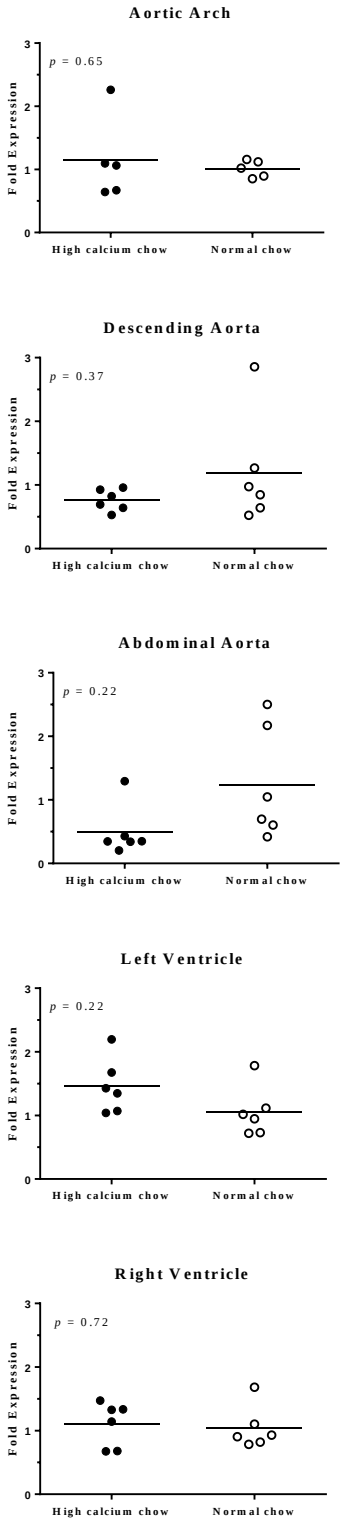


Figure 5.10 Effect of high calcium chow versus normal chow on annexin A6 (*Anxa6*) gene expression in adiponectin knockout mice (n = 6, aortic arch n = 5). p-values are the result of between group analyses using a mixed model two-way analysis of variance (ANOVA), adjusted for multiple comparisons testing (False Discovery Rate).

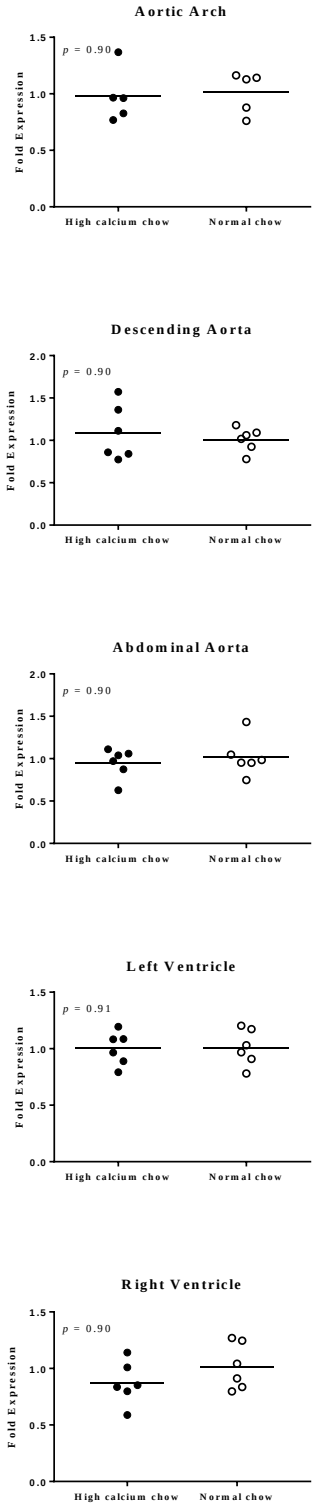


Figure 5.11 Effect of high calcium chow versus normal chow on sodium-dependent phosphate transporter 1 (*Scl20a1*) gene expression in adiponectin knockout mice (n = 6, aortic arch n = 5). p-values are the result of between group analyses using a mixed model two-way analysis of variance (ANOVA), adjusted for multiple comparisons testing (False Discovery Rate).

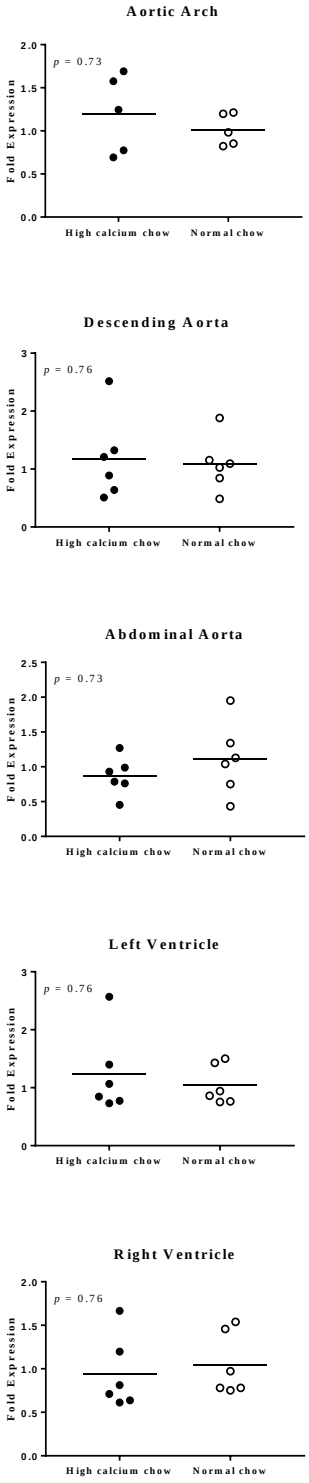


Figure 5.12 Effect of high calcium chow versus normal chow on matrix metalloprotein 2 (*Mmp2*) gene expression in adiponectin knockout mice (n = 6, aortic arch n = 5). p-values are the result of between group analyses using a mixed model two-way analysis of variance (ANOVA), adjusted for multiple comparisons testing (False Discovery Rate).

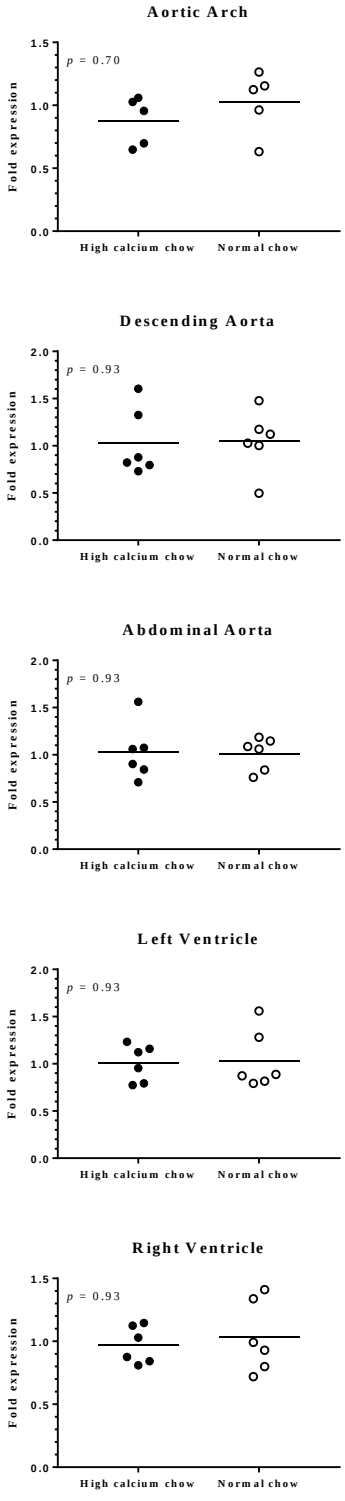


Figure 5.13 Effect of high calcium chow versus normal chow on matrix gla protein (*Mgp*) gene expression in adiponectin knockout mice (n = 6, aortic arch n = 5). p-values are the result of between group analyses using a mixed model two-way analysis of variance (ANOVA), adjusted for multiple comparisons testing (False Discovery Rate).

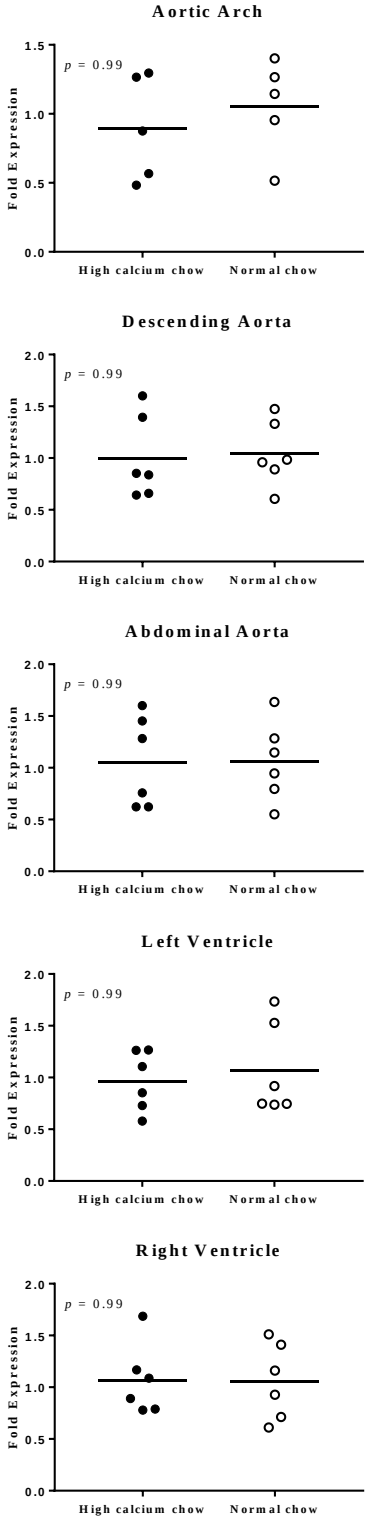


Figure 5.14 Effect of high calcium chow versus normal chow on ectonucleotide pyrophosphatase/phosphodiesterase 1 (*Enpp1*) gene expression in adiponectin knockout mice (n = 6, aortic arch n = 5). p-values are the result of between group analyses using a mixed model two-way analysis of variance (ANOVA), adjusted for multiple comparisons testing (False Discovery Rate).

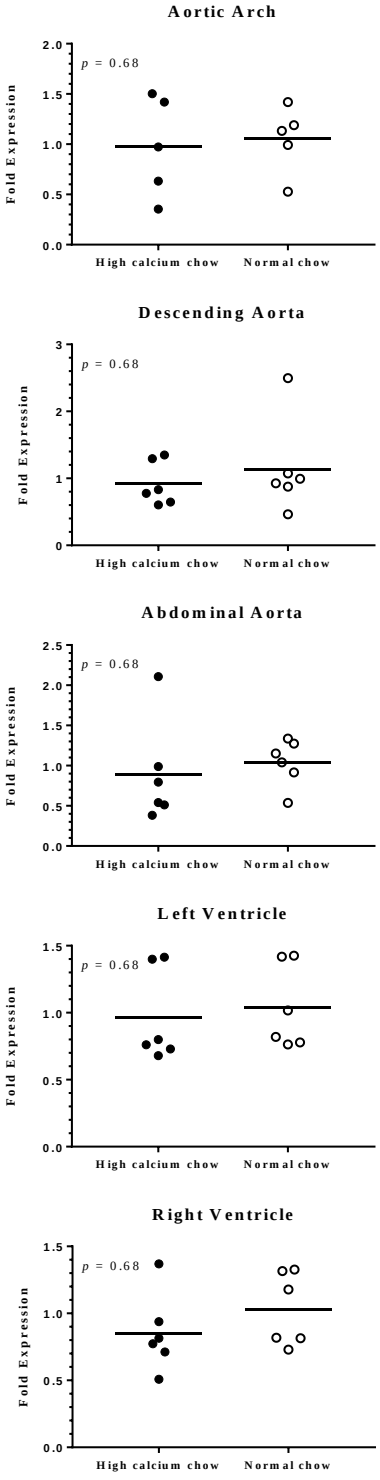
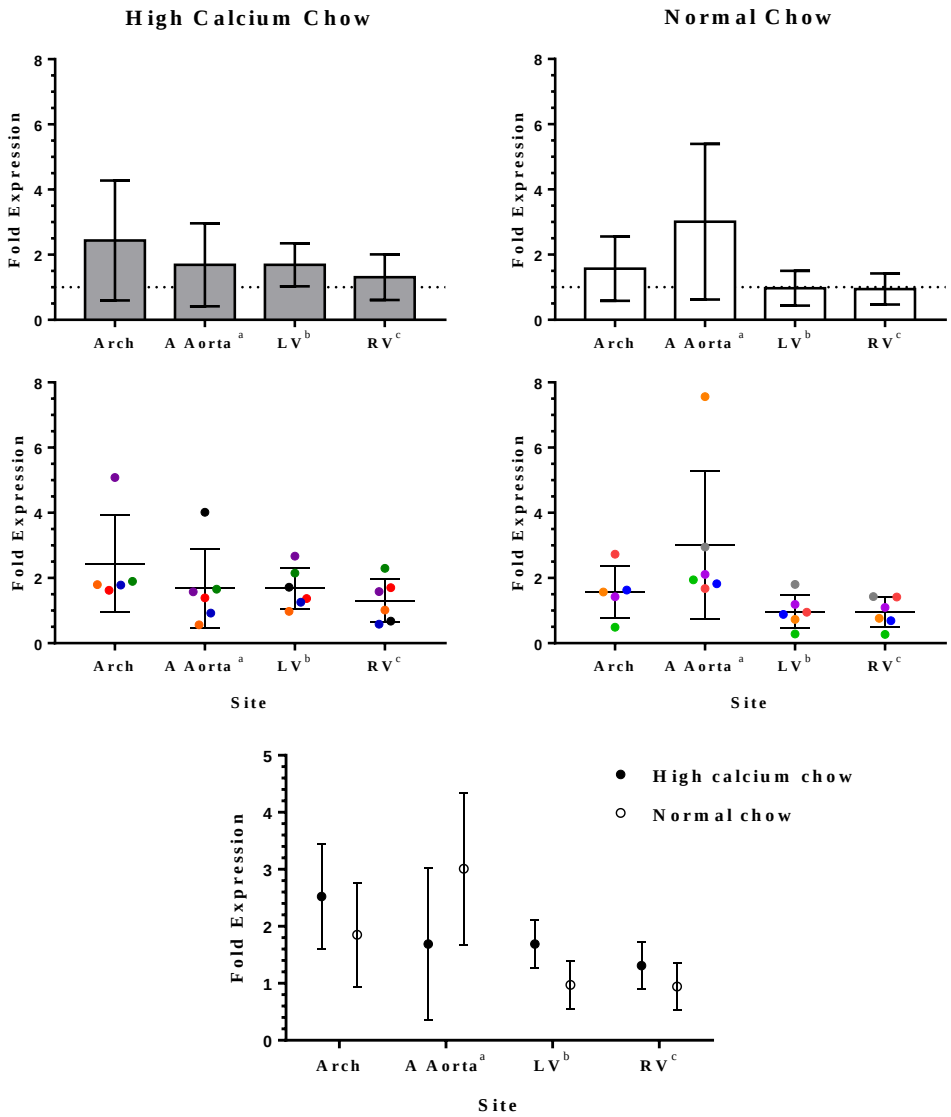


Figure 5.15 Result of high calcium chow and normal chow on *Runx2* gene expression in adiponectin knockout mice (n = 6, aortic arch n = 5). Using the descending aorta as a referent, anatomical sites within each treatment group were analysed using a mixed model two-way analysis of variance (ANOVA). Each colour represents a single mouse. Site variation in gene expression was not observed in *Runx2* expression. Allocation by site interaction p = 0.11. We adjusted for multiple testing comparisons (False Discovery Rate). There were no significant between-group differences at each of the 4 sites. Results are presented as mean, 95% confidence interval.

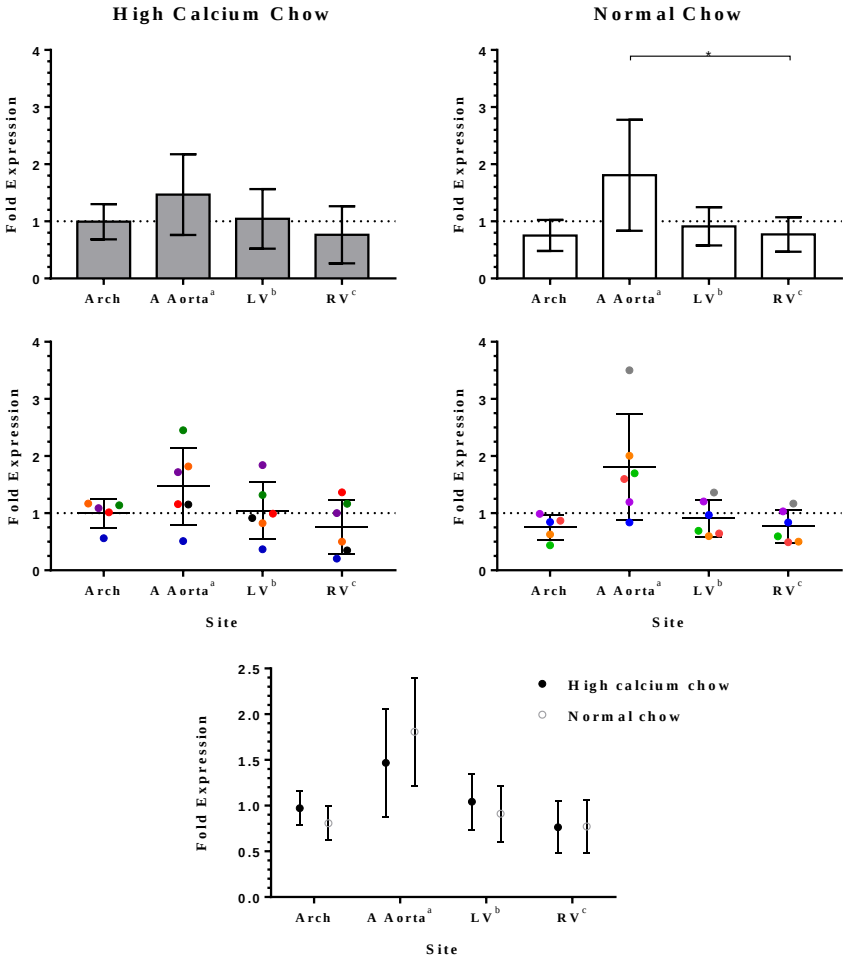


[a] Abdominal Aorta

[b] Left Ventricle

[c] Right Ventricle

Figure 5.16 Result of high calcium chow versus normal chow on sodium-dependent phosphate transporter 1 *(Scl20a1)* gene expression in adiponectin knockout mice (n=6, aortic arch n=5). Using the descending aorta as a referent anatomical sites within each treatment group were analysed using a mixed model two-way analysis of variance (ANOVA). Each colour represents a single mouse. In mice fed normal chow, there was a significant difference in *scl20A1* gene expression in the abdominal aorta compared with the right ventricle (p = 0.05). Site variation in gene expression was not observed in mice fed high calcium chow. Allocation by site interaction p = 0.43. We adjusted for multiple testing comparisons (False Discovery Rate). There were no significant between-group differences at each of the 4 sites. Results are presented as mean, 95% confidence interval.

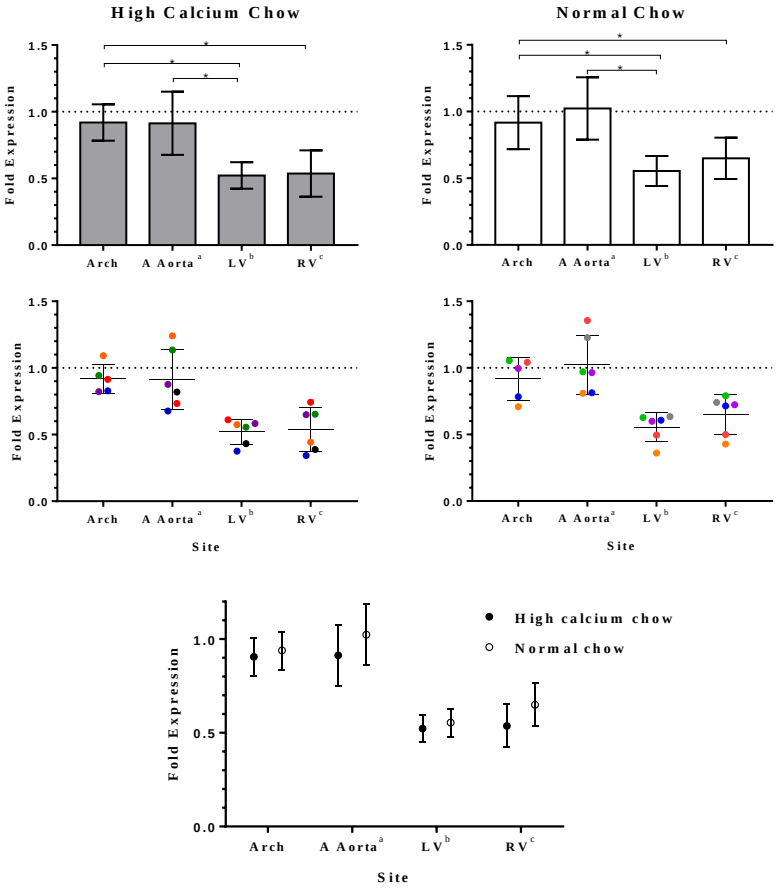


[a] Abdominal Aorta

[b] Left Ventricle

[c] Right Ventricle

Figure 5.17 Result of high calcium chow and normal chow on annexin A6 (*Anxa6*) gene expression in adiponectin knockout mice (n = 6, aortic arch n = 5). Using the descending aorta as a referent anatomical sites within each treatment group were analysed using a mixed model two-way analysis of variance (ANOVA). Each colour represents a single mouse. In the high calcium chow group *Anxa6* gene expression was significantly higher in the aortic arch compared with the left ventricle (p = 0.0001) and right ventricle (p = 0.0039) and higher in the abdominal aorta than the left ventricle (p = 0.0099). For the normal chow group gene expression was higher in the aortic arch compared with the left ventricle (p = 0.0001) and the right ventricle (p = 0.020), and higher in the abdominal aorta compared with the left ventricle (p = 0.0026). Allocation by site interaction was p = 0.082. We adjusted for multiple testing comparisons (False Discovery Rate). There were no significant between-group differences at each of the 4 sites. Results are presented as mean, 95% confidence interval.

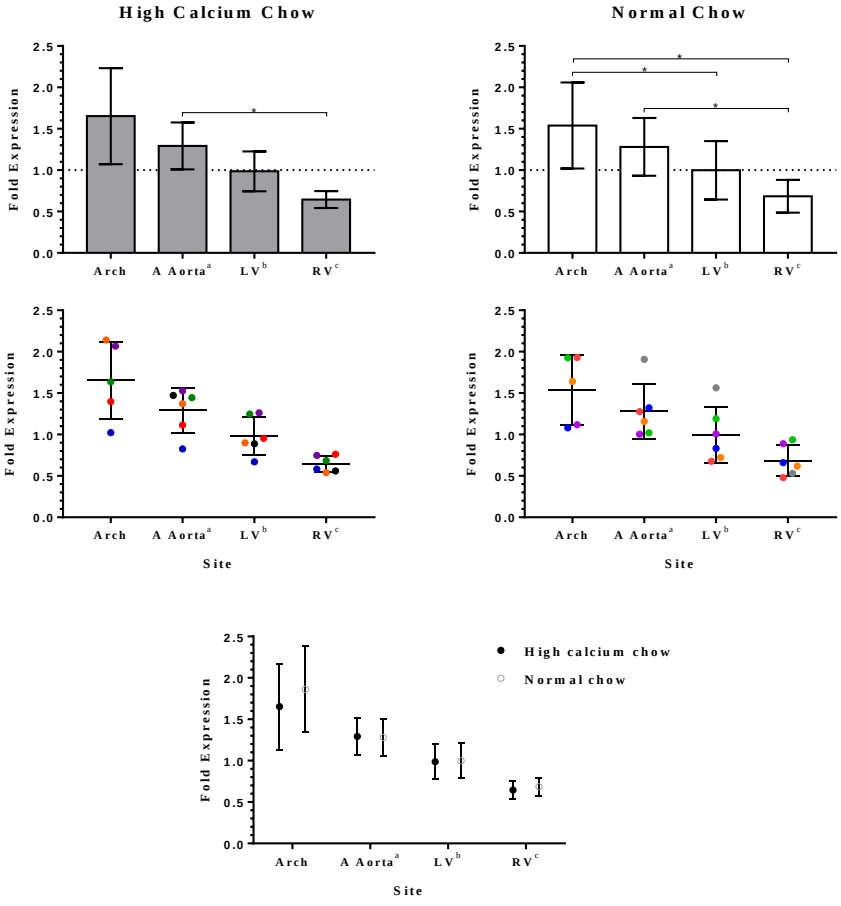


[a] Abdominal Aorta

[b] Left Ventricle

[c] Right Ventricle

Figure 5.18 Result of high calcium chow versus normal chow on matrix metalloprotein 2 (*Mmp2*) gene expression in adiponectin knockout mice (n = 6, aortic arch n = 5). Using the descending aorta as a referent anatomical sites within each treatment group were analysed using a mixed model two-way analysis of variance (ANOVA). Each colour represents a single mouse. In the normal chow group *Mmp2* expression was significantly higher in the aortic arch compared with the left ventricle (p = 0.03) and right ventricle (p = 0.03) and in the abdominal aorta compared with the right ventricle (p = 0.03). in the high calcium chow group *Mmp2* expression was significantly higher in the abdominal aorta compared with the right ventricle (p = 0.018). Allocation by site interaction p = 0.68. We adjusted for multiple testing comparisons (False Discovery Rate). There were no significant between-group differences at each of the 4 sites. Results are presented as mean, 95% confidence interval.

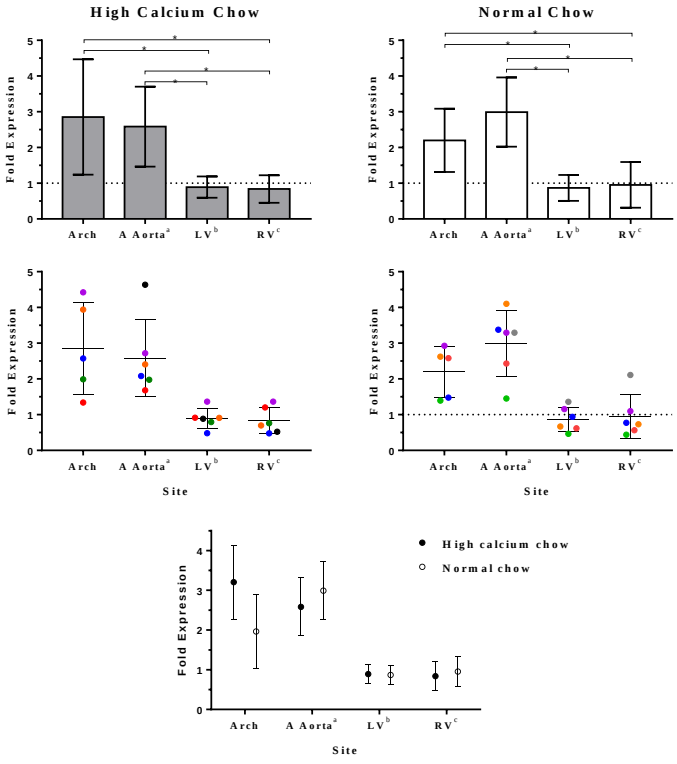


[a] Abdominal Aorta

[b] Left Ventricle

[c] Right Ventricle

Figure 5.19 Result of high calcium chow and normal chow on ectonucleotide pyrophosphatase/phosphodiesterase 1 (*Enpp1*) gene expression in adiponectin knockout mice (n=6, aortic arch n=5). Using the descending aorta as a referent anatomical sites within each treatment group were analysed using a mixed model two-way analysis of variance (ANOVA). Each mouse represents a single mouse. In the normal chow group *Enpp1* gene expression was higher in the aortic arch compared with the left ventricle ($p = 0.0313$) and the right ventricle ($p = 0.0304$) and in the abdominal aorta compared with the left ventricle ($p = 0.0101$) and right ventricle ($p = 0.0061$). This was maintained in the high calcium chow group where *Enpp*1 expression was higher in the aortic arch compared with the left ventricle ($p = 0.0037$) and right ventricle ($p = 0.0045$) and in the abdominal aorta compared with the left ventricle ($p = 0.0019$) and right ventricle ($p = 0.0016$). Allocation by site interaction $p = 0.35$). We adjusted for multiple testing comparisons (False Discovery Rate). There were no significant between-group differences at each of the 4 sites. Results are presented as mean, 95% confidence interval.

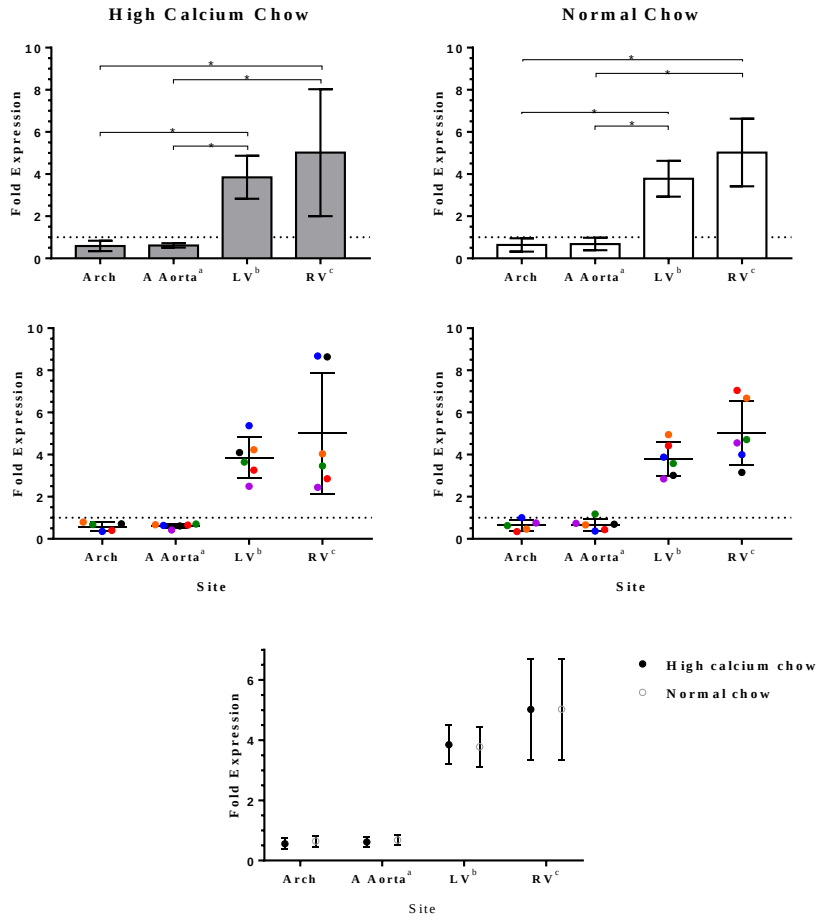


[a] Abdominal Aorta

[b] Left Ventricle

[c] Right Ventricle

Figure 5.20 Result of high calcium chow and normal chow on matrix gla protein (*Mgp*) gene expression in adiponectin knockout mice (n = 6, aortic arch n = 5). Using the descending aorta as a referent anatomical sites within each treatment group were analysed using a mixed model two-way analysis of variance (ANOVA). Each colour represents a single mouse. Results were remarkably similar in both treatment groups with *Mgp* expression in aortic arch reduced compared with the left ventricle ($p < 0.0001$) and right ventricle ($p < 0.005$), and expression in the abdominal aorta also reduced compared with the left ventricle ($p < 0.0001$) and right ventricle ($p < 0.005$) in both groups. Allocation by site interaction $p = 0.97$. We adjusted for multiple testing comparisons (False Discovery Rate). There were no significant between-group differences at each of the 4 sites. Results are presented as mean, 95% confidence interval.



[a] Abdominal Aorta

[b] Left Ventricle

[c] Right Ventricle

Table 5.2 Summary of gene analysis results of the Calcium supplement study and Diet study in Adiponectin knockout mice where the descending aorta is used as the referent and statistical analyses are performed with a mixed model two-way analysis of variance. Variability in gene expression across different anatomical sites is denoted under site interaction either as present (+) or absent (-). All genes showed variability in gene expression in at least one treatment arm, between at least 2 anatomical sites (aortic arch, abdominal aorta, left ventricle, right ventricle) when compared with the descending aorta (refer to figures *-*). Results under allocation by treatment interaction are mean fold changes in gene expression for aorta (aortic arch and abdominal aorta) and heart (left ventricle and right ventricle) compared to the referent descending aorta (1). Treatment allocation significantly altered gene expression across sites only in the Calcium Supplement Study for genes *Scl20a1*, *Enpp1* and *Mgp*.

	Site interaction				**Allocation by site interaction**									
Genes	**Supplement study**		**Diet study**		**Supplement study**					**Diet study**				
					Aorta		**Heart**		**Interaction**	**Aorta**		**Heart**		**Interaction**
	Calcium	**Placebo**	**HCC**	**NC**	**Calcium**	**Placebo**	**Calcium**	**Placebo**		**HCC**	**NC**	**HCC**	**NC**	
Runx2	+	-	-	-	2-2.2	1.6-2.1	0.8-0.9	1-1.4	*p*=0.069	1.7-2.4	1.6-3	1.3-1.7	1	*p*=0.11
Scl20a1	-	+	-	+	0.9-1.3	0.6-1.6	0.6-1.4	1-1.3	*p*=0.041	1-1.5	0.8-1.8	0.8-1	0.8-0.9	*p*=0.43
Anxa6	+	+	+	+	0.9-1	0.9-1	0.5-0.6	0.5-0.6	*p*=0.18	0.9	0.9-1	0.5	0.6-0.7	*p*=0.082
Mmp2	+	+	+	+	1.1-1.7	1.6	0.6-1.3	1-1.3	*p*=0.21	1.3-1.7	1.3-1.5	0.6-1	0.7-1	*p*=0.68
Enpp1	+	-	+	+	1.4-2	1.4-2.4	0.6-0.8	0.8	*p*=0.028	2.6-2.9	2.2-3	0.6-0.9	0.6-0.9	*p*=0.18
Mgp	+	-	+	+	0.8-1.4	0.8-1.1	7.5-11.4	4.1-5.5	*p*=0.0071	0.6	0.6-0.7	3.8-5	3.8-5	*p*=0.97

Table 5.3 Results of post-hoc analysis of gene analysis with the descending aorta as the referent in the Calcium supplement study and the Diet study. Using the ho = 1 directive in SAS each site was analysed to assess whether they differed from the referent (descending aorta). Results are *p*-values.

Scl20a1

	Calcium Supplement Study		**Diet Study**	
	Calcium	Placebo	High Calcium Chow	Normal Chow
Aortic Arch	0.50	0.021	0.96	0.65
Abdominal aorta	0.24	0.37	0.15	0.086
Left Ventricle	0.27	0.47	0.84	0.53
Right Ventricle	0.0048	0.99	0.28	0.11

Runx2

	Calcium Supplement Study		**Diet Study**	
	Calcium	Placebo	High Calcium Chow	Normal Chow
Aortic Arch	0.017	0.0051	0.096	0.19
Abdominal aorta	0.40	0.28	0.22	0.083
Left Ventricle	0.35	0.34	0.043	0.89
Right Ventricle	0.28	0.92	0.31	0.78

Mmp2

	Calcium Supplement Study		**Diet Study**	
	Calcium	Placebo	High Calcium Chow	Normal Chow
Aortic Arch	0.014	0.048	0.035	0.045
Abdominal aorta	0.57	0.092	0.045	0.094
Left Ventricle	0.51	0.21	0.89	0.99
Right Ventricle	0.0012	0.70	0.0003	0.0093

Mgp

	Calcium Supplement Study		**Diet Study**	
	Calcium	Placebo	High Calcium Chow	Normal Chow
Aortic Arch	0.27	0.65	0.0097	0.034
Abdominal aorta	0.24	0.49	0.0002	0.040
Left Ventricle	0.082	0.046	0.0008	0.0004
Right Ventricle	0.014	0.010	0.019	0.0013

Enpp1

	Calcium Supplement Study		**Diet Study**	
	Calcium	Placebo	High Calcium Chow	Normal Chow
Aortic Arch	0.063	0.10	0.033	0.020
Abdominal aorta	0.43	0.11	0.015	0.0032
Left Ventricle	0.51	0.21	0.39	0.39
Right Ventricle	0.025	0.21	0.33	0.86

Anxa6

	Calcium Supplement Study		**Diet Study**	
	Calcium	Placebo	High Calcium Chow	Normal Chow
Aortic Arch	0.17	0.18	0.18	0.31
Abdominal aorta	0.86	0.91	0.39	0.81
Left Ventricle	0.0051	0.0004	<0.0001	0.0002
Right Ventricle	0.0003	0.012	0.0010	0.0021

5.3. Discussion

In this study we were interested as to whether any treatment effect that may be observed with administration of a 120 mg calcium jelly supplement would be mitigated by providing the same dose over 24 hours. Although we found between group differences in serum calcium and phosphate these did not translate into changes in gene expression and therefore within the parameters of this study increasing dietary calcium did not result in measurable abnormalities in vascular calcification or gene expression.

Here we observed that mice in the calcium fortified chow arm gained less weight than those in the placebo group. This is consistent with our tandem jelly supplement study providing evidence in this cohort that an increased calcium intake by either a single daily supplement or over the course of 24 hours provides small but significant decreases in weight. Given the aim of this study was not directed toward the possible weight benefits of increased oral calcium we did not measure body fat nor did we discontinue treatment to observe whether these between-group differences would be lost.

A further consistency was the absence of vascular calcification demonstrated by either inductively-coupled plasma mass spectroscopy or by histology.

Increased dietary calcium led to an increase in serum calcium and a decrease in serum phosphate. This is consistent with other published work albeit after a single high calcium challenge (326,702,713), however, unlike our jelly supplement study this was not accompanied by changes in gene expression. We did observe a reduction in the expression of *Bglap* but given the multiplicity of testing and the relatively weak association, this likely represents a false positive result and is a chance finding. Two possible lines of enquiry emerge: are the differences in gene expression observed in the calcium jelly supplement study a result of the serum perturbations in phosphate and likely calcium or are these merely

confounders? Alternatively, could the constant application of high calcium chow trigger an adaptive response (for example, changes in calcium sensing receptor or annexins (499)) in the vasculature of these mice that has not occurred in those fed a pulsatile application of a calcium jelly supplement therefore mitigating any negative effects? This requires further exploration.

In these studies, we took a 'sampler' approach to gene analysis selecting genes implicated in various mechanisms of vascular calcification. We analysed genes indicating VSMC injury and repair (*ColIa1*), VSMC switch into an osteogenic phenotype (*Runx2, Bglap, Ibsp*), calcification inhibitors (*Mgp*, *Enpp1*), calcium and phosphate channels (*Anxa6*, *Scl20a1*), and a gene implicated in medial calcification (*Mmp2*). It is important to note that these genes have multiple functions and changes in relative gene expression itself is not directly indicative of a propensity for calcification.

Across these genes analysed we verify the presence of anatomical heterogeneity in vascular expression. Although there were no between-group differences visualization of the variation in site expression encourages further enquiry. In the heart, *Mgp* expression was 4-5x higher compared with the descending aorta, and 8-10x that of the aortic arch and abdominal aorta. Matrix gla protein (MGP) is a small protein with nine glutamate residues, five of which undergo post-translational modification to be γ-carboxylated (abbreviated to "gla"). Vitamin-K is required to drive this process and the resultant gla-residues are required for protein activity. In bone formation MGP plays a vital role in chondrocyte viability and function with a biphasic expression pattern during chondrocyte maturation (556). In vascular health MGP prevents medial artery calcification and perhaps also aids in maintaining vascular smooth muscle cell (VSMC) phenotype preventing a switch to that of an osteogenic potential (558,559). It does this by blockade of the osteo-inductive properties of bone morphogenetic

protein-2 (BMP-2) and, in conjunction with fetuin A, by complexing with and sequestering calcium and phosphate within matrix vesicles (MV) to prevent calcification (551,552). Local VSMC must provide this calcification inhibitor, as selective expression in the liver of knockout mice does not rescue the phenotype from vascular calcification (562). The loss of this function is apparent in warfarin therapy where inhibition of the vitamin K dependent formation of gla-residues results in an impotent inhibitor and vascular calcification ensues (563,564). The higher *Mgp* expression in the heart compared with the aorta observed in this study acknowledges a difference of function and perhaps the absence of additional calcification inhibitors as described below. Calcification of the heart would quickly interfere with effective conduction through the electrical circuitry leading to heart block and other irregular rhythms compromising tissue perfusion, whereas the aorta can continue to be an acceptable conduit even in the presence of calcification. Additionally, MGP may also protect myocardial integrity preserving diastolic left ventricular performance and therefore provides at least a dual purpose in the myocardium (714).

A further observation is that *Mgp* expression in the descending aorta was approximately 2x that of the aortic arch and abdominal aorta in both treatment groups. This is a potential mechanism by which the descending aorta is relatively protected from calcification.

Enpp1 expression in the aortic arch and abdominal aorta was 2-3x higher than that of the descending aorta and heart. This gene encodes the enzyme ectonucleotide pyrophosphatase/phosphodiesterase family member 1 which spans the cell membrane in a number of tissue types but is highly expressed in the VSMC, osteoblast and chondrocyte. Mutations in this gene have been linked to generalized arterial calcification of infancy characterized by arterial calcification and fibroproliferative changes with premature death (581,582). Along with ankylosis protein, type III sodium-dependent Pi co-transporter (P_iT-1,

encoded by gene *Scl20a1* in humans), tissue-non-specific alkaline phosphatase, phosphocholine and phosphoethanolamine, it is involved in the careful and complicated regulation of intracellular and extracellular pyrophosphate (PPi, a potent calcification inhibitor) and inorganic phosphate (Pi, mineralization promoter) through the cleavage of ATP (574). It is implicated in the maintenance of vascular smooth muscle cell phenotype, production of sRAGE (soluble endogenous Suppressor of the Receptor for Advanced Glycation End-products), osteopontin release, and the maintenance of serum phosphate and calcium through fibroblast growth factor-23 (574). In the aorta, the balance of these actions weighs against mineralization. A reduced expression in the descending aorta compared with the aortic arch and abdominal aorta could indicate an inherently lower risk of calcification perhaps due to the anatomical absence of major branches and or a reduced vulnerability to phenotypic transformation. Alternatively, *Enpp1* expression in the descending aorta may lean in favour of mineralization as it does in the heart. In the heart cardiac fibroblasts, like vascular smooth muscle cells, have the potential to undergo phenotypic transformation into an osteogenic cell with the subsequent formation of a calcifying matrix. In this setting, *Enpp1* expression is upregulated and the resulting enzyme is used to generate Pi enabling mineralization (586,587). It may be that this lower baseline expression in the descending aorta and heart represents a mechanism to reduce calcification risk.

Anxa6 expression in the aorta was approximately 2x that of the ventricular myocardium in both studies. This likely reflects differences in cell and organ function and therefore machinery. Annexins are a class of regulatory proteins effecting cell responses catalysed by changes in calcium concentration. Calcium binding leads to conformational changes that allow them to reversibly interact with phospholipid membranes where they are involved with membrane organization and trafficking. MV are produced by various cells and play an important role in physiological mineralization. At sites of cartilaginous mineralization they

are secreted by terminally-differentiated chondrocytes forming the initial nucleational core that subsequently continues to propagate along a collagen scaffold (715-717). Annexins are found in matrix vesicles where they facilitate selective enrichment of various proteins vital for potentiation or prevention of apatite formation. They themselves also act as calcium channels and in this way provide calcium for apatite formation within the matrix vesicle (718). Annexins A2, A5 and A6 are major components of mineralizing matrix vesicles from chondrocytes and are absent when matrix vesicles do not mineralize (719).

AnxA6 appears to be important for recruiting AnxA2 and A5 as AnxA6 knockout mice display reduced quantities of the former in matrix vesicles and therefore reduced ability to mineralize at the growth plate (716). It is important to note that this knockout only results in delayed but not abolished mineralization as there is built-in redundancy across some annexins. Bovine and human vascular smooth muscle cells also secrete matrix vesicles and provide one mechanism by which the vasculature is able to calcify (489,490). Matrix vesicles secreted by calcifying bovine vascular smooth muscle cells show increased AnxA2 and A6 content, as well as increased calcium uptake and increased ability to calcify on collagen type I (490). Conversely, when the annexin calcium channel was inhibited, calcium uptake and calcification were impeded.

Physiological concentrations of AnxA6 in the myocardium play a vitally different role compared with the aortic vascular smooth muscle cell. In cardiac myocytes it is important in the calcium fluxes necessary for myocardial contractility. It is implicated in Ca^{2+} release from L-type channels during excitation and involved with Ca^{2+} reuptake necessary for relaxation. Functionally the balance of its actions facilitates intracellular Ca^{2+} clearage with a decrease in percentage shortening of cardiac myocytes and decreased intracellular [Ca^{2+}] by 40% in transgenic mice overexpressing AnxA6 (720). In this way it acts as a negative inotrope

(721,722). Additionally, it has a cardioprotective role in cardiac hypertrophy facilitating atrial natriuretic peptide trafficking and therefore local and distant atrial natriuretic peptide activity and promotes cell viability through stabilization of mitochondrial morphology and dynamics (723). However, under maintained hypertrophic conditions it also facilitates apoptosis of cardiomyocytes (724). Interestingly, 10-fold overexpression of cardiac AnxA6 in transgenic mice leads to myocyte necrosis and inflammation with occasional mineralization (720). It would be beneficial to know whether this mineralization is associated with MV release from cardiac fibroblasts.

Finally, in both studies *Mmp2* expression in the aortic arch was 1.5x higher than that of the descending aorta, and in the diet study expression in the aortic arch was 2x that of the right ventricle. Matrix metalloproteinase-2 (MMP-2) is one of 28 known human matrix metalloproteinases, a family of proteases whose actions include tissue remodelling associated with embryological development, repair following injury, and enzyme activation either directly or via the release of bound components within the extracellular matrix (544,725). MMP-2 has a specific role in the complex looping involved with cardiac morphogenesis (726) and in cardiac remodelling associated with exercise (727). However, one of the known three isoforms is truncated and retained within the cytosol with evidence of localization within cardiac myocytes cleaving vital machinery as well as degrading nuclear material thereby contributing to the cardiac dysfunction following an ischemic-reperfusion injury (544,728,729). MMP-2 also plays an important role in hypertension-induced hypertrophic remodelling of the heart as well as in the aorta where it relieves the vascular smooth muscle cell from the basement membrane through cleavage of type IV collagen thereby promoting a switch to a synthetic phenotype (544,549). In this way it contributes to decreased arterial elasticity and therefore increases cardiac afterload. In rat models of uraemia, MMP-2 is associated with increased osteogenic conversion of VSMC and medial calcification

(547,730). Given the aortic arch is a site of high mechanical stress and consequently a likely site of continued remodelling, it is fitting that this is also the site of highest *Mmp2* expression in our study. Accordingly, the lowest expression is within the right ventricle which is governed by a low-pressure system where injury and repair likely occur at lower rate.

In summary, 120 mg calcium administered over 24 hours as a fortified chow increased serum calcium and decreased serum phosphate in adiponectin knockout mice who received this diet for 32 weeks with no observed changes in gene expression suggesting that an increase in dietary calcium is not harmful to vascular health. Although it is reassuring that an increase in dietary calcium did not negatively affect the vasculature in terms of altered expression of genes implicated in vascular calcification, an understanding of the implications of sera changes and why these did not change the genetic profile would be useful.

In gene analyses nested within each mouse where the descending aorta was used as the referent, we detected heterogeneity of gene expression within the aorta as well as between the aorta and the ventricles of the heart. This provides an interesting preview to the varied and complicated interplay of proteins within their differing local environments.

6. CONCLUDING REMARKS

It seems likely that calcium supplements are responsible for an increased cardiovascular risk in postmenopausal women. The ongoing calcium supplement debate raises some larger questions. As investigators, what should our response be to findings that do not align with popular thinking? More importantly, what is our response to findings that contrast to our own views, views which we have widely propagated? Simply, we should not deviate from our pursuit of excellence and truth. Sometimes, in the light of new evidence, or old evidence brought to light, this means an acknowledgement that I got it wrong, appropriate given that research findings and decisions influence the lives of nations.

Whether or not the multiple re-analyses of the effect of calcium supplements on cardiovascular events shows an increased risk has become redundant. Instead, it has been superseded by a large body of circumstantial evidence indicating that calcium supplements are harmful to the cardiovascular health of postmenopausal women. Oestrogen loss at menopause augments the deterioration in vascular function initiated by aging. Increases in inflammatory cytokines create an undertow to the direct consequences of oestrogen loss on the multiple organ systems, cardiovascular, renal, hepatic, adipose, bone and cerebral; involved in elevating cardiovascular risk. Further contributions by the perimenopausal elevation in serum calcium and PTH, both independently associated with an increased cardiovascular risk, add to an environment that requires little further impetus to insight and augment dysfunction in all systems influencing cardiovascular risk. Therefore, equipped with the knowledge that calcium supplements further increase serum calcium by ~0.1 mmol/L, a rise associated with ~10% increase in cardiovascular risk, in my view the question is no longer, *"Do calcium supplements cause an increase in cardiovascular risk?"* But instead, *"Is it ethical to prescribe calcium supplements to healthy postmenopausal women?"*.

Osteoblasts, along with their fully differentiated counterpart the osteocyte, and vascular smooth muscle cells share many similarities. They are tasked with the continual monitoring of their environment, followed by migration and proliferation where required. They are responsible for the elaboration of an extracellular matrix rich in type I collagen which is a critical component for the scaffold of bone formation, and the structural integrity of the blood vessel. Signalling proteins are similarly sequestered within this extracellular matrix to fine-tune cell responses. In addition, disruption of the extracellular matrix and fragmenting of type I collagen signals a synthetic transformation in the vascular smooth muscle cell and activity in the bone multicellular unit. The striking difference is that the matrix of one is supposed to mineralize, where calcium is used as a substrate for hydroxyapatite; and the other is not, where calcium is involved in the cells' contractile function. The ability of the vascular smooth muscle cell to undergo a phenotypic transformation into a cell of an osteogenic lineage indicates that epigenetic modifications, that occur in the setting of vascular disease, allow access to genes such as *Runx2*, that are transcriptionally silenced in health. That is, phenotype is the result of site-specific relative abundance of proteins that modulate gene transcription. This explains some of the difficulty in translating the findings of single-cell *in vitro* cultures to *in vivo* observations.

In our *in vivo* studies, we have attempted to identify some of the mechanisms whereby calcium supplements might influence a change in vascular phenotype. We did not measure genes implicated in the de-differentiation of vascular smooth muscle cells away from a contractile phenotype, instead we chose to consider genes implicated in the process of vascular calcification. Our findings suggest that calcium administered as a single daily bolus, does produce changes in vascular gene expression but given the exploratory nature of our investigation these will need to be confirmed and followed to ascertain whether changes in gene expression translate to changes in protein synthesis. An important question to address is

whether these changes in gene expression are mediated by elevations in serum calcium. In the calcium supplement study, serum calcium was not elevated in samples acquired 2 hours after the provision of the calcium supplemented jelly. In contrast, mice receiving high calcium chow had an elevated serum calcium but no between-group differences in gene expression were identified. Targeted experiments are required to address this apparent discrepancy.

The vascular smooth muscle cells that populate various segments of the aorta, and coronary arteries have differing embryological origins. This results in embryologically-predetermined responses to different environmental cues, which translates to morphological differences in embryologically defined portions of the aorta. In our *in vivo* study, we observed expected differences in the gene expression between the aorta and myocardium, two distinct cell types. However, we also observed differences in gene expression between anatomical segments within the aorta: the aortic arch and descending aorta, and abdominal aorta and descending aorta. We did not identify differences in gene expression between aortic arch and abdominal aorta. This suggests that, in spite of differing embryological origins, in terms of the genes tested here, the aortic arch and abdominal aorta may be under similar transcriptional control. This is consistent with the observed vulnerability to calcification observed in these two sites.

In secondary analyses of randomised controlled trials designed predominantly to address indicators of bone health, calcium supplements increase cardiovascular risk. This risk does not appear to be altered by important baseline characteristics and reassuringly, does not persist once supplementation is discontinued. Acknowledging, in the context of an already increased risk for cardiovascular disease, that calcium supplements at least could augment cardiovascular risk, would unify efforts to identify the mechanisms by which calcium supplements influence cardiovascular risk in healthy, postmenopausal women.

7. APPENDICES

Appendix 1

Jelly recipe

Ingredients

- 250 ml water
- 25 g sugar
- 25 g gelatine
- 13.75 ml of raspberry flavouring
- 37.5 g of calcium carbonate

Method:

1. Heat water in microwave in large beaker for 3 minutes.

2. Measure out sugar and gelatine in weigh boats. Add sugar to heated water and mix to dissolve.

3. Add gelatine being careful to keep water agitated to avoid clumps. (If clumps do develop these are easily dissolved by using the back of a spoon.)

4. In a smaller beaker measure 37.5 g of calcium carbonate.

5. Take 150 ml of jelly solution and add to beaker containing calcium carbonate.

6. Using multipipettor at “1.2 ml” setting, pipette each jelly into appropriate mould.

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