Exercise and circulating factors associated with skeletal muscle and bone health in older age.

Jessica Piasecki

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School of Healthcare Science

Faculty of Science and Engineering

Manchester Metropolitan University

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Abstract

Muscle mass declines 1-2% every year after the age of 50 years. Contractile forces by muscle exert strains on bone and influence the bone mass and strength. Not only is age a cause for muscle and bone decline, circulating factors can play a role, aiding the signalling between muscle and bone.

Chapter 3 investigates exercise as a way in which we could reduce the loss of whole body bone mineral density (BMD) and muscle mass in older age. Included in this chapter were 38 master sprint runners (28 males, 10 females, mean age 71±7y), 149 master endurance runners (111 males, 38 females, mean age 70±6y) and 59 non-athletic controls (29 males, 30 females, mean age 74±5y). Sprinter hip BMD was 10% and 14% greater than that in endurance runners and controls respectively, but it was difficult to explain this increased BMD by accelerometry or differences in muscle strength. Following on from this, Chapter 4 highlights that there are circulating factors playing a role within the ageing skeleton. Factors dickkopf-1, osteocalcin, osteoprotegrin and sclerostin were identified to be positively associated with whole body bone mineral density in older adults (n=272), with multivariate regression showing body mass index, circulating sclerostin and whole-body lean mass together accounting for 13.8% of the variation with WBMD.

To further investigate the circulating factors, statistical modelling was used to identify those which were also associated with whole body lean mass. Tumour necrosis factor alpha (TNFα) and osteoprotegrin (OPG) were significantly negatively ($r=-0.170$, $p=0.007$) and positively associated ($r=0.140$, $p=0.030$), respectively, with whole body lean mass. With multivariate regression showing height and OPG to account for 45% of the WB Lean mass in older adults. These results, combined with Chapter 4, highlighted OPG as a key molecule associated with both bone and muscle during ageing. Using these findings, in Chapter 6 the interactions between TNFα and OPG were modelled using human myoblast cells in vitro. From the investigations *in vitro* it was clear that OPG is capable of enhancing muscular growth and when incubated with TNFα the myoblasts are able to secrete OPG, providing a protective mechanisms against TNFα. The findings within this thesis can conclude that circulating factors, particularly OPG, are able to interact with muscle and bone and have an influence the decline during ageing. Exercise, particularly, sprinting can help reduce decline in bone health but circulating factors provide a new insight that could help our forever ageing population.

Publications

Page 39: Results in this Chapter have been published in the journal 'Archives of Osteoporosis'.

J Piasecki, JS McPhee, K Hannam, KC Deere, A Elhakeem, M Piasecki, H Degens, JH Tobias and A Ireland.(2018) Hip and spine bone mineral density are greater in master sprinters, but not endurance runners compared with non-athletic controls. *Arch Osteoporosis*, 13 (1), 72

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1.1. Chapter 1: Introduction

Loss of muscle mass occurs progressively after 40 years and the resultant low muscle mass in older age is a recognized disease called sarcopenia [1]. Low muscle mass causes muscle weakness and since the contractile forces developed by muscle exert a strong influence on bone development and maintenance throughout the life span, it is likely that sarcopenia is related to osteoporosis, mainly affecting older adults. Muscle mass and strength decline by around 1-2% per year after 50 years [1]. Osteoporosis affects 3 million older people in the UK, arising as bone remodelling favours resorption of mineralised extracellular matrix over bone formation and increases fracture risk [2].

Evidence suggests that sarcopenic men have a 3-fold increased risk of osteoporosis compared with non-sarcopenic men [3], due in part to lower peak forces transmitted from muscle to bones during physical activities (known as the mechanostat theory) [4]. Despite this evidence from epidemiological surveys, older people with low muscle mass should not necessarily accept a future with osteoporosis and a high risk of bone fractures. Instead, it may be possible even for the small, weak muscles to transfer contractile forces with sufficient osteogenic potential to maintain bone health through highly repetitive eccentric muscular contractions [5]. Such activities would include regular sports training, particularly running or resistance exercises. There has been research to show that exercise during ageing can help slow the decline in bone health [6-8], but there has been very little research into this possibility within the sarcopenic population. There are studies on general older populations to show improvements in the rate muscle loss through exercise, which is discussed later in section 2.1.6. If it proves to be the case, then it will evidence a mechanism through which sarcopenic old may improve their bone health.

It is not only through the direct 'impacts' of muscle forces acting on bone that provide osteogenic stimuli: two-way exchange of circulating factors between muscle and bone can influence cellular signalling in the target tissue and direct growth and maintenance [9, 10]. For instance, bone regulatory factors and cytokines released from osteoblasts during bone formation and osteoclasts during bone resorption are abundant within the extra cellular fluid and enter the circulation where they come into contact with skeletal muscle fibres and satellite cells. These circulating bone regulatory factors have the potential to facilitate cross-talk between bone and muscle tissue, but there has been very little research into possible interactions [10]. Some of the candidate bone regulatory factors possibly related to BMD and acting on skeletal muscle includes: *osteoprotegerin* (OPG) and *osteocalcin* (OC) [11-13] which are secreted by osteoblasts for bone formation; *dickkopf-1* (DKK1) [14] and *sclerostin* [15] are released by osteocytes, which negatively regulates bone formation. Additonally, *osteopontin* [16] (OPN) is another factor released by osteoblasts, osteocytes and osteoclasts to facilitate bone resorption. Tumour necrosis factor- alpha (TNFα) [17] is a pro-inflammatory cytokine, also implicated in bone and muscle remodelling and possibly involved in bone-muscle cross-talk. Very little is known about how these bone regulatory factors act on skeletal muscle and in particular how might this be affected by the process of ageing.

The research completed as part of this PhD study will provide novel insights into the muscle and bone health of exceptionally athletic older adults compared with non-athletic old and will investigate how circulating bone regulatory factors may influence the loss of muscle and bone with ageing. If specific exercise or molecular factors with

osteogenic and/or myogenic potential can be identified, it may lead to new therapeutic targets to help prevent muscle and bone loss in older age.

2.1. Chapter 2: Literature Review

2.1.1 Ageing

The number of people aged over 60 years is growing more rapidly than any other age group. It was estimated that there was 688 million 60 years and over in the year 2006 and by 2050 this number is projected to grow to approximately two billion, with about 20% of those being over 80 years old [2]. The increasing proportions of older people living in our communities is a positive reflection of health and social care advances, but there are also unintended negative consequences as health and physical function decline progressively in old age [2]. Consequently, older people are living for longer with long term conditions impacting negatively on their quality of life. Understanding and combating poor health is a public health priority in order to compress the period of later life spent with disability and to keep older people living independently in their own homes.

Ageing results, as an accumulation over years, from a range of cell and molecular damage. As a result of this damage there is a decline in both mental and physical capacity leading to a greater risk of disease, with the ultimate consequence being death [18]. The deterioration over time will vary from person to person depending upon both genetic and environmental factors [19]. Ageing is considered one of the greatest risk factors for developing musculoskeletal, neurodegenerative and cardiovascular diseases [20]. There have been four main physiological processes identified to contribute to the ageing process at a cellular level; telomere shortening, mitochondrial dysfunction, oxidative stress and cell senescence [21]. Telomeres are repetitive DNA elements found at the end of chromosomes, their role in normal cell function is to protect the DNA ends from damage and degradation. With every cell

division telomeres shorten and eventually, as cells reach a certain age, the telomeres break and initiate cell apoptosis by activation of p53 tumor suppressor protein [22-26].

Mitochondrial dysfunction and oxidative stress can influence one another. Mitochondria are the source of oxidative phosphorylation within cells. As cells age there is an increased amount of reactive oxygen species (ROS) due to continuous mitochondrial DNA damage. The ROS accumulation affects replication and transcription, leading to mutations of the mitochondria DNA, resulting in a further increase in ROS. In turn, this results in a decline in mitochondria function, further DNA damage and ROS production [27], a continuous cycle of damaging events. Thus, it has been deemed that ageing is partly due to an increased accumulation of mitochondrial DNA by the accumulation of ROS, resulting in a decline in respiratory function [28-31].

Senescence is a cell cycle arrest that is induced by certain stressors such as DNA damage. It is induced within replicative ageing of cells. These senescent cells then accumulate within tissues and contribute to the loss of functional ability and lack of regeneration, both associated with ageing [32]. Whilst within cell cycle arrest, the cells also secrete proteins known as senescence-associated secretory phenotype (SASP) [32]. The SASP's disrupt normal homeostasis of the cells, preventing further proliferation, causing more cell death [33].

2.1.2 Ageing and bone tissue

Bone makes up the skeletal system of vertebrates and is capable of adapting according to stresses it undergoes [4]. Its strength and volume adapt throughout life by continuous processes of bone formation and resorption. This bone tissue remodelling

is not maintained in the ageing bone microenvironment, as formation fails to keep pace with resorption [34, 35].

Throughout life, the primary functions of bone are; to provide attachments for muscle tendons and ligaments, to act as a nutrient reserve (particularly calcium), protect the organs and play a role in haematopoiesis [36]. Approximately 75% of bone is composed of compact/cortical bone mass and the remaining 25% is principally accounted for by the inner segment of the bone consisting of interconnecting spicules, forming the trabeculae. This type of bone is called cancellous bone, also known as trabecular bone. The structure of this section is adapted to its function by having a larger surface area allowing bone forming and resorbing cells to have greater chance to contact the bone surface more frequently [36]. The end of bones, known as the epiphysis, are predominantly trabecular bone. The mid shaft of the bones, known as the diaphysis, are much more dense and are predominantly cortical bone. Figure 2.1 shows how the different types of bone are arranged within a typical long bone.

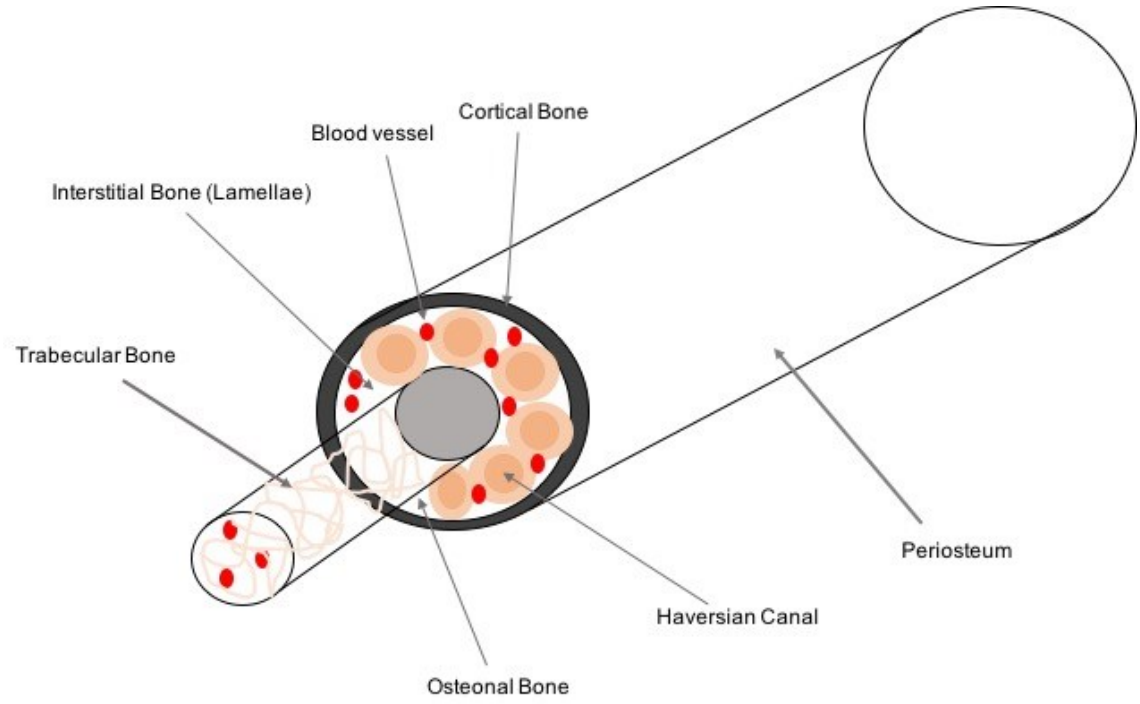

Fig 2.1; Long bone cross sectional view, showing the internal structure of the bone. Adapted from [37].

The mass of bone is maintained by the balances of two types of bone cells known as osteoclasts and osteoblasts. Osteoblasts are involved in formation of bone whilst osteoclasts are involved in bone resorption. The interaction of osteoblasts and osteoclasts at the bone surface are demonstrated in figure 2.2. Osteoclasts are derived from haemopoietic stem cells. The osteoblast cells are derived from mesenchymal stem cells along with stromal and bone lining cells. For a steady state of bone homeostasis, it is important that the balance between osteoblasts and osteoclast action are maintained. Bone resorption occurs at a much higher rate than does bone formation, a small increase, hence an imbalance of the action, in osteoclasts could then cause bone loss [36, 38]. Bone resorption by osteoclasts takes just 30 days and the bone remodelling cycle takes 3 months [38].

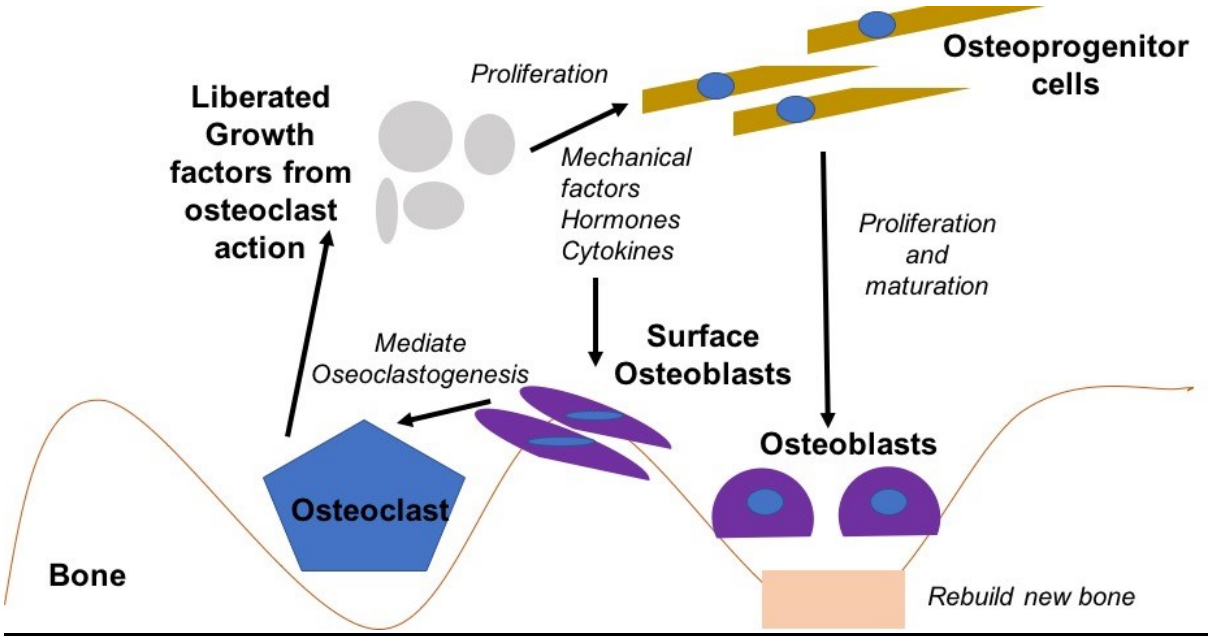

Figure 2.2; An example of the interactions between osteoblasts and osteoclasts involved in bone remodelling processes. Adapted from [38]

The majority of bone mass is accumulated by the age of 20 years old [39]. Approximately 26% of resultant adult bone mass is accumulated during the final two years surrounding peak bone growth velocity, which approximates the amount of bone lost, later in life, during post-menopausal years, for women in particular [39-42]. Males usually have a greater BMD than females, as shown in twin studies [43], the decline in BMD starts at different time points for males and females. For males, the decrease begins around the age of 40 and females, around the age of 30 years [44].

Ageing is associated with a loss of bone mass and strength [45] due to a greater rate of resorption (via osteoclasts) than formation (via osteoblasts), and a reduction in the force of muscle contractions. Ageing bone can also be influenced by increased free radicals as a result of oxidative stress [46], in particular advanced oxidative protein products [47] which have been shown to be negatively correlated with femoral BMD. Another study has highlighted hydrogen peroxide (H_2O_2) or xanthine/xanthine oxidase

(XO), as free radicals, which resulted in inhibition of differentiation markers in the bone osteoblastic cells, MC3T3-E1, as well as the marrow stromal cells, M2-10B4, whilst leading to increased oxidative stress [48].

Within a clinical setting this age-related decline may be identified by a reduction in BMD and/or an increased rate of bone fracture. As the skeleton naturally reduces in strength and density over time, it is more likely to succumb to the skeletal disease, Osteoporosis, which is often associated with menopause and ageing. Osteoporosis is characterized by low bone mass along with 'micro architectural' deterioration of bone tissue which increases the fragility of the skeleton and, therefore, the increased likelihood of a fracture [49]. Osteoporosis arises as the balance of bone remodelling favours resorption of mineralised extracellular matrix over formation [2]. It affects around 3 million people in the UK alone [2, 50]. The main problem of Primary Osteoporosis is the continued breakdown of trabeculae structure within the bone. For females, post-menopause, there is a significant decline in oestrogen availability, contributing to the decline in BMD with age. For ageing males, sex hormone binding globulin has the ability to inactivate testosterone and oestrogen, also contributing to the decline in BMD [51-54]. Secondary Osteoporosis arises due to co morbidities or disease such as Cushing syndrome [55] and long-term glucocorticoid therapy [56]. Life style factors such as poor diet, particularly low in calcium, under nourishment, smoking and excessive alcohol are all detrimental to bone health [51, 57].

Osteoporosis was defined using the T-Score, which is a measurement of bone density in $g.cm^2$ compared to a bone density measurement of a healthy 30-year-old adult of the same sex. Osteoporosis is classed as 2.5 standard deviations below the T score,

typically using sites of the hip, spine or forearm [58]. Within research Z-scores tend to be used which are similar measures to that of the T score, except the Z score is compared to an age matched person, rather than a 30 year old adult. In 1994 it was identified by the World Health Organisation (WHO) as an established, defined disease affecting more than 75 million people across Europe, Japan and the US [59]. Currently the assessment of bone mineral density (BMD) by dual X-ray absorptiometry (DXA) is the preferred measurement available in a clinical setting [59] and osteoporosis usually remains undiagnosed until a fracture arises and imaging is subsequently performed [60]. The hip and lumbar spine are sites most likely to experience the most severe effects of osteoporosis due to the quantity of trabecular bone within the vertebral bones [58], and the hip being the initial point of impact if a person falls sideways [58]. However, when imaging these areas other factors such as scoliosis, calcification and vertebral fractures may impair the ability to diagnose osteoporosis [58, 59]. To that end, more recently, the osteoporosis diagnosing criteria has changed to use the femoral neck only, along with an international reference standard per age, using the same reference for both men and women. This is deemed suitable as the risk of fracture at the femoral neck at any age is similar in men and women [59, 61-64]. Another imaging technique used to assess bone health is peripheral quantitative computed tomography (pQCT). Using pQCT, the inner structure of the bone can be assessed, as shown in several studies [7, 8, 65], comparatively to just the aerial view of the bone as seen in DXA imaging. However, pQCT can only be applied to the limbs and it is not able to assess the bone mass or geometry of the hip and spine, which are the main sites affected by osteoporosis in older age.

Falls are also more likely to occur in older age, [66, 67]. This, combined with a weaker skeleton, predisposes the old to an increased fracture rate. Hip fractures in particular are associated with a high mortality rate [53, 58, 62] and are a significant cost to the current national health service [58, 59]. In 2010, within Europe, it was estimated that the cost of fragility fractures, was 37 billion Euros. Of this cost, 66% attributed to incidents, 29% for fracture care and just 5% on pharmacological interventions [59, 68]. This total cost is expected to increase 25% by the year 2025 [59, 68].

There are both non-pharmacological and pharmacological treatments suggested to help management of osteoporosis [60]. In the early stages of the disease, non-pharmacological interventions are recommended, such as increasing dietary calcium and vitamin D intakes, 1000-1200mg of calcium is the current recommended daily intake for elderly men and women [69]. Along with smoking cessation, the limitation of alcohol consumption and increasing weight-bearing exercises [69-72]. Weight bearing exercises are discussed later in this thesis. Pharmacological therapy is usually prescribed following a fracture, aimed at reducing the likelihood of any further fractures. These medications are usually anti-resorptive, decreasing the rate of bone resorption such as bisphosphonates, or others are anabolic, which increase bone formation, such as teriparatide [69, 73]. Long term use of these drugs does come with potential side effects, such as acid reflux, gastrointestinal problems, poor renal function, jaw necrosis which affects approximately 1 in 10,000 [74], and osteosarcomas [75]. The rate of osteosarcomas is relatively low, with 2 out of 430,000 patients presenting in 2009 [69, 75]. Alternative ways of managing osteoporosis are always being investigated.

2.1.3 Exercise and Bone

Strains the bone receives during exercise are compression, tensile and torsional forces as well as shear stress. All these strains and stresses can be applied to the same bone at the same time during exercise. The specific strain and amount of strain that is needed to stimulate bone remodelling is still yet to be defined, as is the specific recommendations of type and duration of exercise in ageing needed to maintain bone health [76].

Exercise across the life course can help to prevent osteoporosis in later life [77]. Exercising during youth ensures higher peak bone mass is achieved [78, 79] as well as delaying the onset of age related decline in BMD [80, 81]. Bone strength is determined by its mass and density. Regular activity is linked with an increase in bone density [82]. Forces acting on bone from muscle contractions or other impact activities stimulate bone formation, while reduced loading with sedentary living or microgravity will decrease bone formation and can stimulate bone resorption. The physical activity level of an individual is therefore an important factor in bone health and a vital reason to encourage participation in sport.

Bone's adaption to exercise is mediated by cellular mechanotransduction [83]. Mechanosensors that are found throughout bone cells can change their structure following a strain on the bone. These changes stimulate the Wnt/β-catenin signalling cascade [83] to be activated either by stimulation of a bone transcription factor (RUNX2) [84] or by cross talk with parathyroid hormone (PTH) and/or bone morphometric proteins (BMPs) [85-87]. Bone adapts to exercise through the muscle contractions [4, 88] associated with physical activity, impacts and ground reaction forces [76, 89].

There have been numerous studies conducted to understand the impacts of exercise and physical activity on bone health during ageing. Master athletes are those whom compete and train at an elite level, and usually have done so since a very young age. Several studies have looked at the master athletes as an example of 'better ageing'. Wilks et al (2009) demonstrated the benefits of master athletes, firstly showing that sprint, middle and long-distance runners all have a greater tibial bone strength compared to controls [7]. This study consisted of 106 sprinters, 52 middle distance runners, 93 long distance runner, 49 race walkers and 75 age matched sedentary people. The tibia of master athletes was around 70mm^2 greater in the athletes and the tibia had a trabecular BMD 50mg.mm^3 greater than controls, in both male and female athletes. This was only found at the impacting tibia bone, results for bone strength at the radius did not show any obvious advantages in the athletes compared to controls. Wilks further investigated discipline specific advantages on bone health for these master athletes [8]. pQCT highlighted that diaphyseal bone mineral content, cortical area and polar moment of resistance were a much larger size in the sprinters. This increase then declined gradually over the distances, middle then long distance runners, race-walkers and controls having the lowest values. Sprinters had approximately 16% greater values than controls, similarly the periosteol circumference of the tibia was approximately 6% greater in sprinters than controls. The females tending to have a much greater difference to the controls than males. Again, very few differences between groups were found for the radius. These results show there is a bone expansion seen within the athletes allowing for greater muscle forces to be produced, in turn inducing a greater osteogenic effect on the bone, helping to enhance bone strength. From these studies it is clear to see that bone is able to adapt

throughout the life-span but this adaption is site specific and should be taken into account when prescribing exercise to older people. Whilst both of these master athlete studies demonstrate the advantages of training on bone, there was a large age range, of master athletes used for the study (33-94 years) making it difficult to know at what ages the differences become apparent.

Numerous randomised controlled trials have been performed to investigate the impact of exercise on the ageing skeleton and to identify the most appropriate form of activity for the ageing population. These studies have shown both positive and negative outcomes. Woo et al., (2007) [90] compared Tai Chi and resistance training in a community dwelling ageing population. The study involved 90 men and women (total n=180) aged 65-74 years. After 12 months, training three times a week of either Tai Chi or resistance, the women showed a moderate attenuation in the loss of hip BMD, measured using DXA, (approx. 0.01% loss), with no changes in men, compared to a non-exercising group (2.25% loss). Whilst this outcome is positive for women, there are only minor benefits from 12 months of training. Duckham et al (2015) showed similar findings, [91]. Two falls prevention programmes were compared with regular care, the Otago Exercise programme (OEP) and the Falls exercise management (FEM) that involved both home and group exercises. Within this study there were a total of 319 participants with an average age of ~72±5 yrs. The FEM involved 39 mins of group exercise a week and 30 min of home exercise per week, whilst the OEP group completed 58 mins a week of home exercise per week, with each intervention being carried out for 24 weeks. The aim of the falls programmes was to increase bone and muscle strength and to reduce the likelihood of falls in the future. There was no difference in femoral neck BMD or bone mineral content (BMC) between any of the

groups, nor was there an increase in BMD or BMC at any of the skeletal sites measured, using DXA. It was suggested that the exercises within the programmes did not exert a strain great enough to stimulate the bone and prevent further resorption within ageing. Whilst both these studies had high compliance rates ~80%, it may be that using the DXA as an outcome measure cannot pick up small positive bone changes but a pQCT may show internal structure or geometry changes of the bone that cannot be picked up using DXA. More studies are needed using these methods alongside DXA to see if this is the case.

Several other studies have investigated the effects of a variety of exercises on bone health, Rantalainen et al (2011), [92], showed bilateral hopping for 12 weeks to have no effect on changing levels of circulating bone markers CTx, CICP and bALP, markers of bone turnover. These particular circulating bone markers have a short half-life and so changes can be hard to detect. Beavers et al (2017), [93], compared two groups of males and females aged 69±3.5 years (n=123). 63 participants underwent 5 months of resistance training, 3 days per week, whilst the remaining 60 people underwent 5 months of aerobic training, 3 times a week. The resistance training involved 8 body exercises, each was done in 3 sets of 10 reps at 70% of the participant's 1 repetition maximum. The aerobic training involved walking for 30 mins at 65-70% of maximum heart rate. Using DXA, BMD was measured at baseline and 5 months after the intervention. The results of this study showed the resistance training group to have no changes in their BMD, the aerobic group showed a significant decline in BMD (-7%). These findings further highlight the need for the exercises to exert high force strain on the bone and of a long enough duration to show positive effects on the bone health.

There are some studies that have showed positive effects on bone health through set exercise programmes. Elsisi et al (2015), [94], used a sample of elderly women aged ~65±3.0 years, either assigned to circuit weight training or low pulse magnetic field for 12 weeks, both interventions showed a significant increase in BMD and BMC of the lumbar spine, femoral neck and femoral trochanter ($p<0.0001$). Marques et al ,[95], and Tolomio et al [96], used combined exercise interventions of aerobic, strength and balance training. Marques et al (2011), [95], had a group of male and females (total n=60, aged~69±5.5), with the intervention conducted over 8 months, Tolomio et al (2008), [96], focused on osteoporotic women (n=49) with the intervention lasting 20 weeks. Marques et al, showed an increase of $0.1 g.cm^2$ at the femoral neck BMD, with no change in controls. Tolomio et al, improved all strength parameters of the lower limb, measured using phalangeal quantitative osteosonography. Both interventions showing positive effects on the BMD and bone parameters measured. These findings can be compared to studies of bed rest or space flights whereby there is no impact or physical activity and just 6 months of space flight can result in a 10% loss of BMD [97-99].

These studies highlight that there is inconclusive evidence to show that exercise has beneficial effects on the ageing skeleton. The majority of resistance training studies, discussed in this section, seem to show beneficial effects to bone density during ageing. It seems that the interventions not involving resistance training must produce strains on the bone that are of a level high enough and longer duration to produce an osteogenic response. More studies are needed to specify the exact type of exercise and duration that will help to slow down the loss of bone strength during ageing, as well as the different effects of the exercise between clinically relevant sites.

These conclusions are also confirmed by a number of systematic reviews and meta analysis conducted to assess the effect of physical exercise on bone density. Bolam et al (2013), [100] conducted a systematic review assessing the effects of physical activity on bone health but focusing on the effects on meddle-age and older men. Their analysis included 8 randomised controlled trials, of which the interventions were; walking (n=2), resistance training (n=3), walking and resistance training (n=1) and resistance training + impact activity (n=1).Out of the 8 studies, 5 had a score below 50% on the authors quality scale and the interventions varied in terms of frequency, intensity and duration. Six of the studies showed positive effects on BMD, while two showed no significant changes. The resistance training studies showed to be the most likely to produce a response in BMD, whereas walking alone had very little benefit. They also concluded that more high quality intervention studies are needed to establish the precise optimum exercise regime. Similarly in randomised controlled trials for post-menopausal women, another systematic review (n=17) by Yeh et al (2018) [101] has shown exercise to have a moderate effect size on bone mineral density on the lumbar spine in post-menopausal women (5 randomised controlled trials, 311 participants, SMD=0.38, 95% CI:0.08-0.68). Whilst a moderate effect size was reported only one of the studies showed a significant change in BMD (p=0.011). Again, demonstrating the need for consistency in randomised controllled trials. A systematic search was also performed, initially, for this section, results and search criteria of which can be found in appendix 1.

Monitoring levels of habitual physical activity can also be a limiting factor within these studies, as habitual levels can vary greatly within a population, one way could be to

use accelerometry measurements [102]. However, it should be noted that some accelerometers may only register vertical impacts and not horizontal components of acceleration [103]. Further research is needed to test whether overall (horizontal and vertical) accelerations are associated with bone adaptations observed in different types of physical activity.

2.1.4 Muscle

Skeletal muscle is made up of fibres that usually run the total length of the muscle or, for pennate muscle, they run diagonally from superficial to deep aponeuroses. Within each fibre there are myofibrils and within myofibrils there are units known as sarcomeres. A contraction of a muscle occurs at the sarcomere level. Filaments of the sarcomere (actin) slide across thicker filaments (myosin dimers). The more myofibrils that a fibre contains, the greater the maximal force [104]. Muscles are innervated by motor neurons; these neurons carry the signal to the muscle to instigate contractions. A motor unit describes a single motor neuron and the muscle fibres that are innervated by the branches of that neuron [105]. A whole muscle contraction occurs when the individual motor units are activated. An action potential passes along the motor neuron axon and its branches to reach the neuromuscular junction. At this junction, acetylcholine is released from vesicles at the axon terminal into the synapse cleft. The acetylcholine then binds to receptors on the cell surface of the motor end plate on the muscle fibre [105], stimulating an action potential to travel along the sarcolemma and through t-tubules. The end result causes a release of calcium from the sarcoplasmic reticulum into the muscle fibre sarcoplasm [104]. The calcium binds with troponin, causing a conformation change in tropomyosin, exposing the myosin binding site on actin, allowing the myosin head to bind with actin. Adeonosine triphosphate (ATP) is hydrolysed to liberate energy needed for the ‘power stroke’ as

the myosin head rotates to 'pull' actin towards the centre of the sarcomere to generate force, or tension. ATP binding with myosin allows myosin to detach from actin. This process continues for as long as calcium is available in the sarcoplasm, and in turn, calcium will continue to be released into the sarcoplasm for as long as action potentials continue to arrive at the neuromuscular junction (assuming no fatigue occurs).

There are three main fibre types that are each associated with different types of exercise based on the motor units and their differing capacities. Type 1 fibres are found in low threshold motor units, they have a low velocity of shortening during contraction, are enriched with mitochondria and myoglobin and are fatigue resistant. These fibres benefit from endurance performance or other prolonged activity. Type 2a muscle fibres usually have a larger cross-sectional area than type 1, along with a faster velocity of contraction and intermediate levels of mitochondria. Type 2x fibres have the largest cross-sectional area, fastest velocity of shortening and lowest mitochondrial content but higher glycolytic activity. The type 2x fibres fatigue quickly but generate high power and are found in the highest threshold motor units. These fibres are associated and used more within power and sprint training [106].

The maintenance of muscle mass is regulated by the balance of protein breakdown and protein synthesis. This protein synthesis is regulated by the physical activity that is undergone by the muscles as well as the type of food that is ingested. Leucine is an essential amino acid, found within the make-up of certain proteins. This amino acid has the ability to inhibit the enzymes, classed as proteasomes, that are responsible for the breakdown of protein. During ageing, muscle becomes less able to respond to

physical activity and protein ingestion, and may require a greater amount of protein per day to elicit an anabolic response [107-110].

2.1.5 Ageing and Muscle

Ageing has a great effect on skeletal muscle. At the age of 40 years old, lean muscle mass begins to decline. After the age of 50 years old, muscle mass declines approx 1-2% per year, with a total decline of around 25% between 50 and 75 years old [111-114].

The strength of muscle also declines and the rate of decline increases with advancing age [115]. Power can be determined as muscle force x velocity of contraction and declines of power are greater than those of maximal force because muscles also slow. The velocity at peak power has been shown to decline around 18% during middle age and then a further 20% between the ages of 80 and 89 years. Other studies have also shown muscle size and strength to decline 68% from young (25 years) to old (71 years) [116]. The loss of muscle mass with age can be partly attributed to an acceleration in the atrophy of fast twitch muscle fibres (type 2x), these fibres have a reduction in cross sectional area at an increased rate than the type 1 fibres. The proportion of type 1 to type 2 fibres, however, remains the same [117, 118]. It has been suggested that the action potential resulting in a release of calcium into the sarcomere, becomes impaired leaving a less tense muscle, deemed as muscle weakness [119, 120]. Specific tension of single fibres decreases with old age and results in a reduced muscle specific force, further contributing to weakness in old age along with low muscle mass [121]. As muscle mass declines, connective tissue and fat replace the area previously

occupied by the muscle fibres. This further hinders the maximum contractile force of the muscle [105].

The loss of muscle fibres with age is associated with death of motor neurons. Studies on human cadavers have revealed that those aged around 75 years old have around 30% fewer motor neurons innervating lower limb muscles [122-124], and within the vastus lateralis there have been shown to be as much as 40-50% fewer motor neurons [117]. These findings have recently been supported using electromyography measurements indicating a loss of 30-50% of motor units by the age of 70 years old [105, 116]. Once muscle fibres become denervated, they may not all atrophy and die off, some may be reinnervated by remaining nearby axons. As a result, the motor unit becomes larger, but throughout the muscle as a whole, the number of motor units reduces [116, 125, 126]. This reinnervation also results in fibre type grouping [126, 127].

There are other factors that play a role in the decline in muscle size and strength. Satellite cell numbers reduce with age. Satellite cells are usually responsible for the repair of muscles in response to injury and damage. A reduction of these with age results in a slower response of the muscle to injury and a delayed repair [128-130]. An in-balance between the rate of protein synthesis and the rate of protein breakdown is more likely to occur as, in the elderly, food intake and appetite tend to decline [131]. Additionally, levels of certain hormones, such as oestrogen, progesterone and cortisol, change with age which can also have an effect on the muscles. The decline in human growth hormone reduces the amount of growth and repair. Cortisol levels also rise and can increase skeletal muscle fibre atrophy, whilst testosterone and oestrogen

reduction has been associated with decreased muscle mass and protein synthesis [25, 132, 133].

In 1989, Rosenburg proposed the definition of sarcopenia as the “progressive decline of muscle mass and strength”. Sarcopenia is normally associated with increased risk of falling and fracture, loss of independent living, hospital admission and increased mortality [134, 135]. An updated definition of sarcopenia characterises the syndrome as progressive loss of muscle mass and strength associated with an increased risk for physical disability, poor quality of life and death [136-138]. There are many ways in which sarcopenia may be defined including, but not limited to, muscle mass, muscle strength and physical performance (walking speed or grip strength) [137, 139]. Current estimates suggest approximately 200 million people worldwide will be affected by the year 2050 [140]. The prevalence of sarcopenia increases with age, amongst 60-70 year olds approximately 5-13% are affected by sarcopenia, when aged over 80 years old, the proportion affected ranges from 11-50%, with a greater amount of men effected compared to women [141-143].

Ageing also triggers a pro inflammatory response characterised by higher levels of circulating tumour necrosis factor-alpha (TNFα) and C-reactive protein, which is linked with a gradual decline in physical activity, presenting as low grade chronic inflammation [144]. Studies have shown that sarcopenia is accompanied by increased levels of inflammation factors TNFα and Interleukin-6 (IL-6), these factors have a catabolic effect in the long-term, accelerating muscle and bone tissue declines [134, 145, 146].

In one population study, a sample of sarcopenia patients had a morbidity rate of 17% in males and 19% in females [144]. The progression towards sarcopenia ultimately involves alterations in skeletal muscle protein turnover; when the rate of muscle breakdown exceeds the muscle protein synthesis [144]. Interventions targeting the inflammatory responses that are associated with sarcopenia have not been successful and do not seem to be able to manage the atrophy of muscle, nor has it been explored in full detail [144, 147].

As sarcopenia is a multifactorial condition, no single factor intervention study has been shown to have great effects. A combination of intervention and life style changes are what is needed to help reduce the loss of muscle size and strength in later life. The main focus of these interventions should target nutritional strategies that ensure sufficient energy and protein intake, maintain physical activity and reduce low grade inflammation [144, 146]. A summary of the changes in muscle mass, motor units and fibres during ageing, as discussed in this section, are shown in figure 2.3.

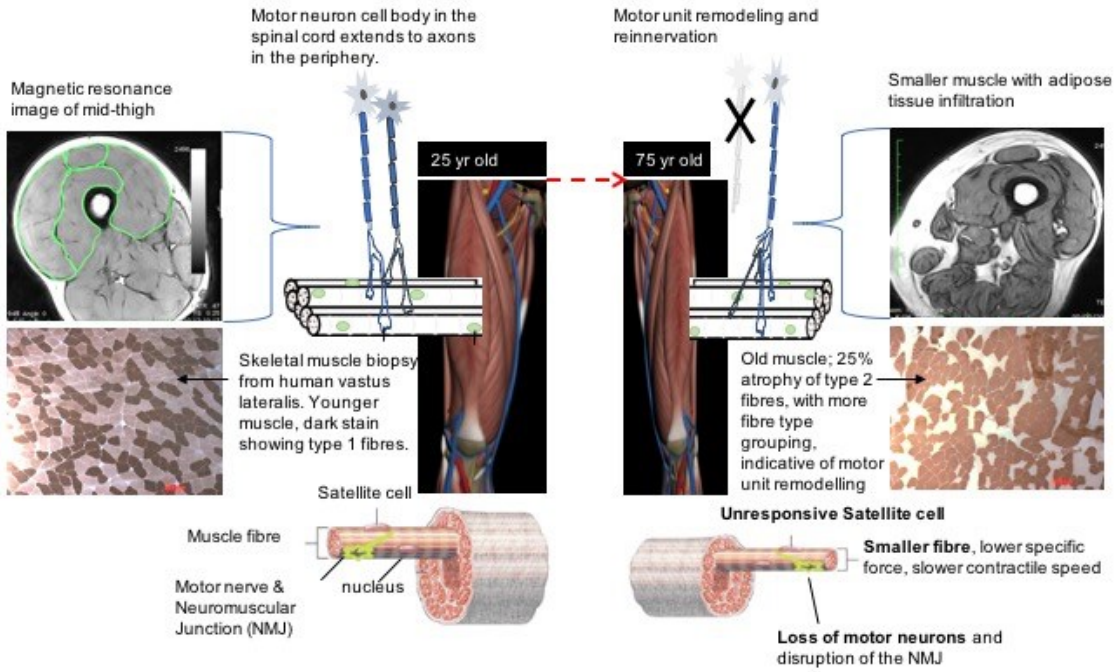

Figure 2.3; A comparison between young and old muscle, showing motor unit remodeling, reinnervation, reduction in muscle size and fibre type grouping. Adapted from [105].

2.1.6 Exercise and Muscle

Physical activity has positive effects on muscle mass in ageing, in particular reducing the prevalence of sarcopenia in older age. Interestingly, it has been shown, within samples of local communities that sarcopenia is associated with a higher mortality rate and the greatest predictor of this mortality rate is a low physical function. Landi et al (2016), [148], investigated the elderly population within the area of Sirente in Italy. 364 subjects were used for the study, all aged ~ 85 (median range 80-100) years. These community members were assessed over the course of 10 years, of those Sarcopenia was identified in 103 participants. Additionally, 253 deaths were recorded, 10 of which were sarcopenic, 162 were non sarcopenic. Of those deaths, subjects with a low physical performance level had a greater mortality rate. Similarly, Mijnarends et al (2016), [149], investigated a large community within the Reykavik area, with 2309 participants aged 66-93 years. Over a five-year period, the incidence of sarcopenia had more than doubled within the sample, rising from 7% to 16%. Those reporting a higher level of physical activity had a significantly lower likelihood of becoming sarcopenic with older age. Studies such as these highlight the importance of improving physical function as a way to reduce the rate of sarcopenia and related mortality.

As exercise impacts on bone, section 2.1.3, master athletes have also been used as an example ageing population to demonstrate the effects of exercise on muscle. There are clear differences between the muscular strength of master athletes and age matched controls [150, 151]. The benefit, however, is only achieved within power and strength trained master athletes, endurance trained athletes show no difference in muscle strength and size to that of age match controls [152-154]. The extent to which

consistent training over a number of years effects muscle in ageing has been well reviewed by McKendry et al (2018), [150] in which the authors highlighted that, due to the wide variety of methods applied, it cannot be concluded if muscle mass and morphology is maintained better in trained master athletes. Although, most studies showed no differences between groups of old and young, sprint and endurance, in muscle fibre architecture [155, 156]. This highlights the need for consistent methods for measurements of muscle ageing within this specific population group, and also the need for specific training definitions within ageing populations.

Numerous studies have been conducted to investigate the varying effects of the different types of exercise and physical activity has on muscle and sarcopenia during older age. It is well known that resistance training increases muscle protein synthesis, strength and power without the need for any specific dietary interventions [157]. Elderly populations have been shown to respond to resistance training interventions, as clearly identified by Laussen et al (2015), [158], in which community dwelling older people, aged 70-85 years, took part in a 6 month progressive resistance exercise program. The training was carried out three times a week progressing from two sets of 10 repetitions at 80% of their maximum to 3 sets of 12 repetitions at 80% of their maximum. Significant improvements were identified in all measures of physical function, stair climb, chair rise and short physical performance battery testing (SPPB; a combination of three balance stances, 5x chair rises and a timed walk at their usual pace, denoting a total score that gives an indication of physical function [159]) . The SPPB score improved by 2 points, the chair rise became 9 seconds quicker and stair climb improved by 1 second ($p<0.03$). Some studies have shown different improvements between male and females undergoing the same exercise program. For

example, Hanson et al (2009), [160], used tests such as the stair climb test, rapid walk and timed up and go as assessments of physical function, aimed at mimicking activities of daily living (Total n=59 average age~69±7 years). Following a 12-week strength training program. Women (n=27) improved their walking timed test, by 0.5 seconds, but not the stair climb, whereas men (n=23) improved their stair climb test, by 0.3 seconds, but not the walking time test. These studies clearly highlight the physical and muscular function improvements that can be made in old populations via exercise. When reporting changes in sarcopenia prevalence it should be noted that there are several ways in which sarcopenia can be diagnosed, making it difficult to confirm the effects of the interventions [161].

Resistance training has shown to be effective in the older person, using different forms of delivery. Both, low volume resistance training and high-volume resistance training can be effective, as was shown by Reid et al (2008), [162]. Subjects aged ~78±8 years old were divided into two groups, one performing high velocity resistance training (n=22)at a lower load, the other performing low velocity but at a higher training load (n=23). The intervention was carried out for 16 weeks, with training sessions completed twice a week. The result showed both groups improved their leg strength (13% for high velocity and 16% low velocity) and frailty assessment scores, as noted previously these assessment methods may come from an extensive range, (1.4 and 1.8 point improvement, respectively), with no differences between the two types of resistance training. Another study, Nunes et al (2016), [163], has also shown that low volume exercise (3 sets per exercise) and high volume (6 sets per exercise) can both increase muscular strength of the leg extensors, by approximately 20kg, over a 16 week period, with participants aged approximately ~61±(54-68 median range) years.

Aerobic training has been shown to have some effects on reducing inflammation as well as over time reducing body fat percentage [164-166]. Nordic walking is designed to work all muscle groups, walking using poles which involve the upper body muscles as well as the lower limbs. A sample of osteoporotic or osteopenic women aged 68.7±4.43 years were randomly assigned to a control group or an activity group [167]. The activity group performed Nordic walking for 60 mins 3 times a week for 12 weeks, the control group continued with no additional intervention. During the 12 weeks, the activity group showed significant improvements in their skeletal muscle mass index and the strength of the knee extensors and flexors. The peak torque of the knee extensor improved in the exercise group from 97Nm to 108Nm, and the flexor from 42Nm to 51Nm, over the 12-week period. There were no changes within the control group. However, the walking did not improve hand grip strength. Indicating greater forces may be required at the forearms to stimulate an overall improvement in muscle strength. Another study has shown that aerobic training can improve muscular function. This study has shown that the muscular function is improved by remodelling the contractile properties of muscle [168]. Using an elderly population of women aged 71±2 yrs, 12 weeks of cycle ergometer training, carried out 3-4 times per week for 20-45 mins showed an increase in size of type 1 fibres (16%) and an elevation (21%) in peak power of type 1 fibres. Clearly, aerobic training does have its place in additionally increasing muscle mass, power and strength amongst older populations. However, there is speculation that long term aerobic endurance training [169, 170] is more detrimental to both bone and muscle health in older age than resistance and power training [8, 164, 165].

Various studies have shown that the combination of both resistance and aerobic training provides the best means to enhance the aging musculoskeletal system [164-166]. Results of a large longitudinal study were reported by the LIFE study group [171] in 2017. This involved 1,635 sedentary men and women aged ~78±5.4 years being enrolled in a randomised controlled trial, and then being followed up over the course of 2.6 years. General day to day physical activity was monitored using accelerometry data and self-reporting. The participants all had functional limitations, and they were randomised into a structured moderate intensity physical activity programme combining walking, resistance and flexibility, or a general health education programme. At baseline and 6,12 and 24 months the 400m gait speed and short physical performance battery test (SPPB) were performed as outcome measurements. Overtime, the time for gait speed and SPPB score improved significantly ($p<0.001$), which were associated with increases in physical activity by an increasing number of accelerometry counts. These improvements in physical activity also reduced the onset of disability within the sample. Such a study highlights not only that combined interventions can instigate physical improvements but also that a sedentary population of older age is still able to respond significantly to such an intervention.

These studies have all shown that exercise is able to benefit the older population and improve muscular health and reduce the likelihood of onset of sarcopenia. Whilst there is a very large set of studies that all highlight the benefits of exercise there is still, yet to be defined, the most beneficial type and duration of exercise to reduce the prevalence of low muscle mass and onset of sarcopenia in old age. Most studies, as identified in this section, have used general ageing samples whom may already be active and/or healthy, and importantly, there are very few long-duration training

programmes reported in the literature. Similar conclusions have been drawn from systematic reviews, one particular review by Vlietstra et al (2018) [172] revealed that knee extension strength ($p<0.01$), timed up and go ($p<0.0001$), appendicular muscle mass ($p=0.04$) and leg muscle mass ($p=0.04$) all showed significant improvements in response to the exercise interventions, within older adults with sarcopenia. This review evaluated 32 full texts, but only 6 randomised controlled trials were found within the 32 full texts. This further demonstrates the positive effects of exercise on muscle mass and strength as well as highlighting the need for more randomised controlled trials with consistent methods. Lee et al (2018) [173], achieved similar results with their systematic review, which included seven randomised controlled trials and three cross sectional or longitudinal studies. Muscle mass, muscle strength and physical performance all improved significantly in the sarcopenic subjects. However, there was little consistency in the measurement of sarcopenia. A systematic search was also performed, initially, for this section, results and search criteria of which can be found in appendix 2.

2.1.7 Muscle and Bone Interactions

The mechanostat theory states that bone adapts to increased mechanical loading, (impact exercise), by increasing size and strength [4, 82, 174] with reduced mechanical deformation showing major declines in bone mass, size and strength [174]. There have been numerous heritability studies that estimate between 40-80% of the phenotypes of the skeleton can be attributed to genetics, similar proportions have also been reported for muscle traits [175-178]. Given these high proportions of genetic influences that underlie both bone and muscle, it is highly likely that there is a shared genetic component between muscle and bone [175-178].

Individually, both bone and muscle are able to act as endocrine organs, secreting substances into the circulation which, in turn, may act on other organs. In the late 1970's it was first recognized that muscle can act as an endocrine organ particularly in response to injury [179]. Myostatin was the first myokine to be identified [180, 181] and is now known as an inhibitor of skeletal muscle cell growth. IL-6 is also a known 'myokine', defined by Pedersen et al, as a cytokine released by the muscle cells, usually in response to inflammation and injury, leading to increased plasma levels of IL-6 [179]. Pedersen and her group also showed that contracting muscles led to a 19-fold increase in plasma concentrations of IL-6 comparatively to resting muscles [182]. There are numerous other myokines that have since been identified, IL-5; which was studied as having a role in cross talk between adipose and muscle tissue [183] and IL-7 has be shown to have possible effects on satellite cells during differentiation of myogenic cells [183]. Some studies have shown Irisin to convert white fat into brown fat [184], IL-15 has been shown to be involved in reduction of adiposity and studies using mice over expressing the gene for IL-15 are associated with having a higher bone mineral density [185].

In 2003, Winkler et al provided evidence that osteocytes function as more than just a specified bone cell, it can also act as an endocrine organ, regulating bone density by secretion of circulating factors [9]. Another factor, osteocalcin, is released by bone and can circulate in the blood where it can interact with substances from the liver and adipose tissue, to enhance energy metabolism. The way in which osteocalcin interacts with the substances released from the liver and adipose tissue may pre-dispose people to obesity, diabetes and osteoporosis [10]. Some other factors released by bone cells are osteoprotegerin (OPG), expressed by osteocytes and osteoblasts. OPG can cause a

reduction in the number of osteoclasts produced by binding receptor activator of nuclear factor kappa-B ligand (RANKL) [11]. Dickkopf-1 (DKK1) [14] and sclerostin, are, primarily released by osteocytes [15], and negatively regulate bone formation. DKK1 and sclerostin have both emerged as therapeutic targets to tackle osteoporosis [186]. Fibroblast growth factor 23 (FGF23) is produced by a wide variety of cell types, including osteoblasts and osteocytes. Once FGF23 is released into the circulation it is able to act on the kidney to increase excretion of phosphate and reduce production of 1-25 OH Vitamin D [187]. Osteopontin (OPN) is an extracellular matrix protein released by osteoblasts, osteocytes and osteoclasts and facilitates bone resorption [16].

Together, the paracrine and endocrine properties of bone and muscle have led to the proposal of 'bone-muscle cross talk'. The interaction between muscle and bone can be highlighted during exercise as muscles increase their function to power movements. Uptake of glucose and fatty acids rises significantly to provide energy needed to maintain muscle contractions [13, 188]. Osteocalcin (Ocn) is a factor that is now readily researched and a good example of how bone and muscle may interact [13, 189]. Genetically altered mice with their gene encoding osteocalcin silenced, have been shown to run for around 30% less time than wildtype mice [13]. The same low exercise capacity was also found in 3-month-old mice lacking the osteocalcin receptor (Gprc6a) [190]. Ocn(-/-) mice knockouts and Osteoclacin mice knockouts in osteoblasts only, both showed a similar decline in exercise capacity indicating the absence of a bone to muscle signalling event, highlighting the importance of the bone-muscle cross-talk. Il-6 also rises during exercises and enhances the ability to carry out the exercise and can interact with muscle and bone (as well as many other tissue types) causing different substances and molecules to be released to enhance exercise capacity (see Figure 2.4).

In summary, osteocalcin increased the uptake and breakdown of glucose and fatty acids in myofibres. Then, in turn, the rise in IL-6 secretion from muscle during the exercises triggered by osteocalcin allows glucose to be mobilised from the liver and fats to be broken down into fatty acids. Finally, IL-6 through regulation of bone resorption increases the amount of bioactive osteocalcin produced [13]. This is summarised in the figure below: adapted from [13].

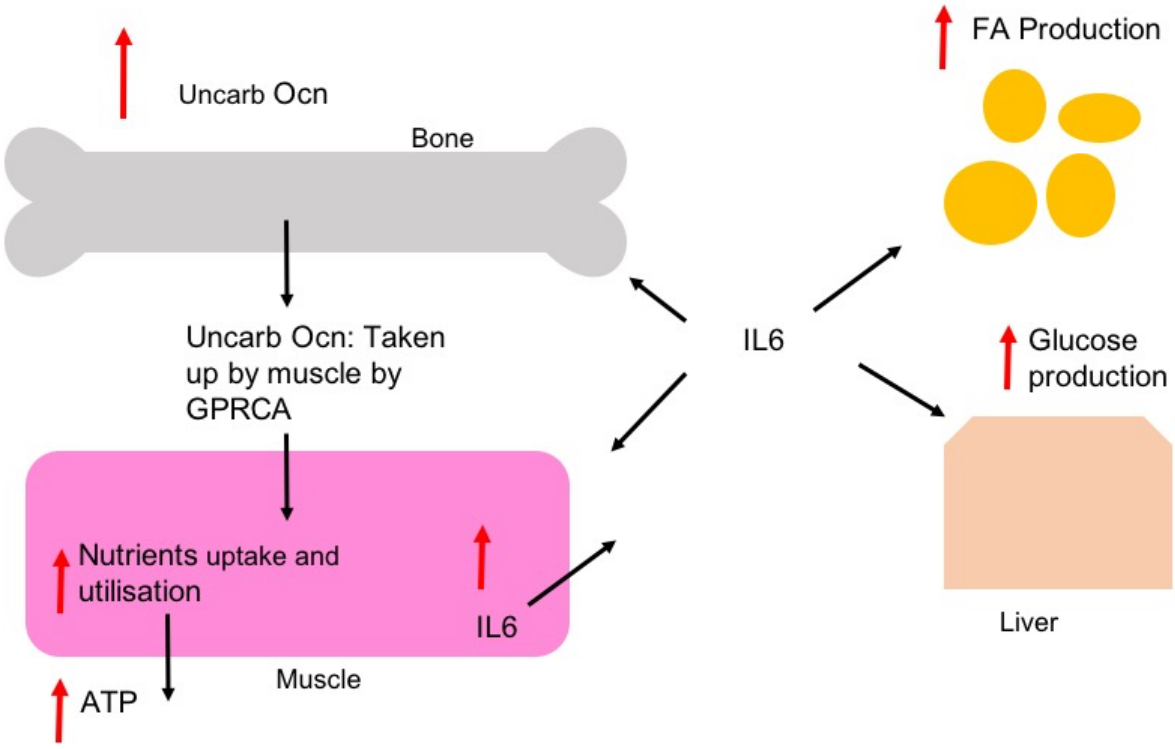

Figure 2.4: An illustration of how Osteocalcin remodeling in myofibres can be accountable for most of the IL-6 released during exercise. An example of bone and muscle cross talk.
Uncarb Ocn: Uncarboxylated osteocalcin, FA: Fatty acid, GPRC6A: Osteocalcin receptor, IL-6: Interleukin 6, ATP: Adenosine triphosphate. Red arrow indicates an increase in production, black arrow indicates direction Adapted from [13]

There has been further evidence to the interaction between muscle and bone through injury. Healing of fractures are much delayed if there is muscle damage alongside the fracture, [191-194]. With open fractures in mice with paralyzed muscles, healing is delayed, whereas if the fracture is covered with a functioning muscle, healing time is significantly reduced, indicating that the muscle is able to secrete factors to the

surrounding bone, decreasing healing time [195]. This has not only been shown in mice but in a clinical setting in humans with open fractures of the tibia [196].

2.1.8 Osteoporosis and Sarcopenia

As discussed in earlier sections, both sarcopenia and osteoporosis exist in similar population groups, those being elderly, sedentary and in particular for osteoporosis post-menopausal women. It is now well known that sarcopenia and osteoporosis frequently co-exist within older populations, the common links between the two comorbidities are shown in figure 2.5. In studies using older men, it has been shown that the measures of appendicular lean mass, 15-20% of the variability is explained by BMD [3, 197]. Similarly, with postmenopausal women, several studies have shown a strong positive correlation between whole body lean mass and whole-body BMD [198-201]. Using the mechanostat theory it would be expected that changes in bone mass are mediated by changes to the muscle strain (mechanostat). Therefore, numerous exercise intervention studies into muscle or bone have been conducted, as reported in sections 2.1.3 and 2.1.6, in an attempt to identify the best type and necessary duration of exercise to improve muscle and bone health during ageing. There is still yet to be defined a clear exercise programme for the ageing population.

While the role of direct forces applied through muscle tendons onto bone is reasonably well established as a mechanism of bone growth or decline, the role of circulating factors mediating muscle and bone development, or decline remains unclear. The circulating bone factors previously mentioned, however, are still yet to be fully established and some have speculative roles amongst bone-muscle cross talk, especially within ageing.

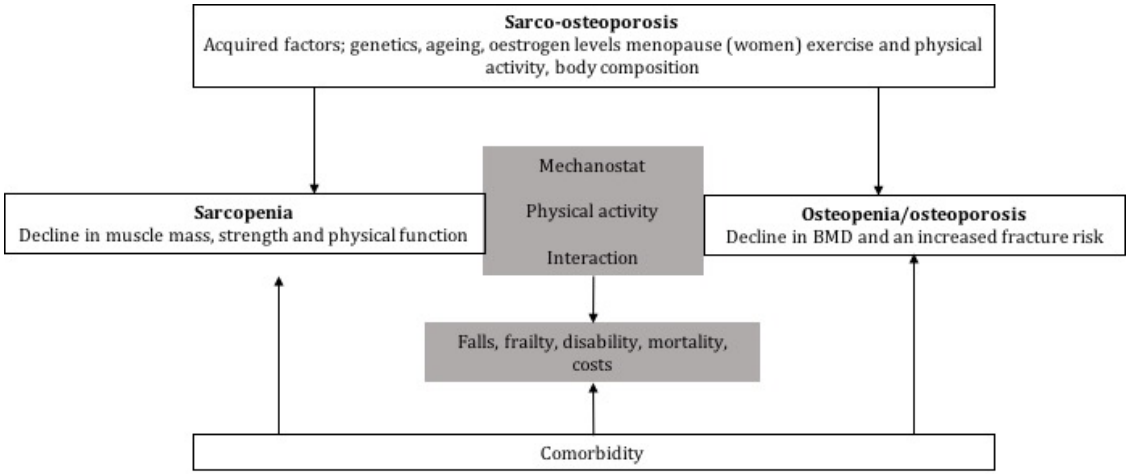

Figure 2.5; Adapted from [127]; How sarcopenia and osteoporosis may come to co-exist in older age.

The research completed as part of this PhD study will provide novel insights into how exercise and circulating bone regulatory factors may influence both bone and muscle health within older age. If specific regulatory factors with osteogenic and/or myogenic potential can be identified, it may lead to new therapeutic targets to help combat both osteoporosis and sarcopenia in older age. If we are able to identify the most beneficial type of exercise for ageing muscle and bone this will help to inform the general population on how to slow down the decline of muscle mass and bone strength in older age.

2.2 Aims and Objectives

The overarching aim of the PhD project was to investigate how exercise and circulating bone regulatory factors influence both bone and muscle health in older age. This was achieved through four objectives:

1. Investigate the relationship between BMD and lean mass in athletic older people, taking into account accelerometry data, the training age and type of athlete (sprint or endurance).
2. Identify the circulating bone remodelling factors associated with BMD in healthy young and older adults.
3. Identify the circulating bone remodelling factors associated with whole body lean mass in healthy young and older adults.
4. Using the selected circulating factors identified in objective 2 and 3, investigate the responses of cultured human immortalised myoblasts after exposure to these factors.

3.1 Chapter 3: Hip and spine bone mineral density are increased in master sprinters compared to endurance runners and controls.

3.1.2 Abstract

Purpose: The relationship was examined between prolonged participation in regular sprint or endurance running and skeletal health at key clinical sites in older age.

Methods: Participants included 38 master sprint runners (28 males, 10 females, mean age 71±7y), 149 master endurance runners (111 males, 38 females, mean age 70±6y) and 59 non-athletic controls (29 males, 30 females, mean age 74±5y). Dual X-ray absorptiometry was used to assess hip and spine bone mineral density (BMD), body composition (lean and fat mass), whilst jump power was assessed with jumping mechanography. In athletes, vertical impacts were recorded over 7 days from a waist-worn accelerometer, and details of starting age, age-graded performance and training hours were recorded.

Results: In ANOVA models adjusted for sex, age, height, body composition and jump power, sprinter hip BMD was 10% and 14% greater than that in endurance runners and controls respectively. Sprinter spine BMD was also greater than that in both endurance runners and controls (11% and 6%, respectively). There were no differences in hip or spine BMD between endurance runners and controls. Stepwise regression showed only discipline (sprint/endurance), sex and age as predictors of athlete spine BMD, whilst these variables and starting age were predictive of hip BMD.

Conclusions: Regular running is associated with greater BMD at the fracture-prone hip and spine sites in master sprinters but not endurance runners. These benefits cannot be explained by indicators of mechanical loading measured in this study including vertical impacts, body composition or muscular output.

3.1.3 Introduction

To follow on from the findings in the literature review, the different effects of sprint and endurance training on bone mineral density were investigated. Clinically relevant sites, the hip and spine, were designated as the two bone outcome measures. To explain any differences between groups accelerometry data was collected along with muscle power and body composition, addressing the first objective for this PhD.

Bone adapts to the mechanical loading it experiences during everyday physical activity (PA) and exercise, with higher impacts associated with intense PA being advantageous for bone strength [4, 82, 174]. Older people are usually less active than young and what activities they do engage with tend to be low impact and therefore of little benefit to bone [76]. This age-related decline in physical activity likely contributes to declining bone strength. Indeed, positive associations have been reported between PA levels and bone strength in older adults [76, 202], suggesting that exercise is an effective way to improve and maintain bone mineral density (BMD) and bone strength in older individuals. Interventions designed to improve bone strength through exercise training have shown some significant improvements in adults, there is still a proportion of which have failed to show clinically significant effects [203]. A possible explanation for this is that bone adaptation in adults is slow and effects of exercise may take several years to fully manifest [6]. There is also uncertainty over the types of activities that are potentially osteogenic.

Master athletes offer a model to examine associations between long term exercise training and bone strength, and have the added advantage that comparison can be made between different disciplines to determine which activities are more osteogenic. In young adults, the benefits of regular exercise have been suggested to depend upon

the type of activity, being greater in high impact activities such as sprinting whereas little benefit is evident in lower impact activities such as walking, cycling or swimming [204-206]. This may also be the case for older adults. For example, master cyclists have a higher incidence (80%) of osteopenia and lower hip and spine BMD than non-athletic controls (50%) [169]. In contrast, male and female sprinters had 15% and 18% greater trabecular BMD in the distal tibia than non-athletes [207], whereas benefits in male and female endurance runners were 7% and 9%, respectively. It remains unclear whether the benefits of sprint and endurance running are also observed in older age for the hip or lumbar spine, fractures of which represent a major disease burden. In a small study of master athletes (n=26), total body, arm, trunk pelvis, legs, thoracic and lumbar spine regional BMD were greater in sprint athletes than controls with no advantages evident in endurance runners [208]. Previous studies have omitted comparisons with controls, considered younger athletes, been limited by small sample size or not investigated these regions [170, 207, 208].

To the extent that observed associations between discipline and BMD reflect a response to exercise, different benefits of distinct running events on BMD are likely to be related to differences in skeletal loading by muscle and reaction forces between those activities. For example, the larger reaction [209, 210] and muscle forces in sprinting could explain the greater benefits to bone in sprint compared to endurance running. Direct assessment of vertical impacts and indirect indicators of muscular loading (lean mass and muscle power) in sprint and endurance runners would provide relevant information to test this hypothesis.

It was hypothesised that both athletic groups have greater bone strength than controls, with the largest advantages in sprinters. In addition, to the extent that any observed differences were a consequence of exercise participation, it was expected that the larger bone advantages in sprint than in endurance athletes are attributable to differences in physical activity (accelerometry data) and muscle mass and function. To investigate this, we compared hip and spine BMD between master sprinters, master endurance runners and non-athletic controls. We also examined differences in the number of vertical impacts and indicators of mechanical loading such as body composition and muscle power, and through ANCOVA and multiple linear regression models examined to what extent these could explain group differences.

3.1.4 Materials and Methods

3.1.4.1 Study Design

Master athletes (MA) were recruited at nationwide athletics competitions as part of a multiple cohort study named "VIBE" and included male and female athletes aged ≥60 years currently competing in sprint, middle or long distance running and in the 12 months preceding recruitment had competed at regional level or higher. Regional ethics approval (14/NW0275) was obtained prior to the study and written informed consent was obtained from all participants.

MAs were classified as sprinters (28 male and 10 female,) if competing in events less than 800m in distance, or endurance athletes (111 male and 38 female) if competing in events greater than or equal to 800m in distance. Each athlete completed a questionnaire to determine demographics, lifestyle, their past physical activity behaviours and physical activity at the time of wearing the accelerometer. The

questionnaire data allowed the athletes to be grouped according to years trained consecutively: 1) those training all of their life through childhood; 2) those training since 18 years old, 3) those training since 30 years old, and 4) those training since 50 years old. Mean age-graded performance (AGP) was determined by taking the athlete's highest ranked performance within the last two years, and expressing it as a percentage of the world record for that age and distance. AGP ranged from 77-92% across the cohort, indicating a high level of performance relative to respective age group records. For example, a marathon of 3 hours and 30 minutes at the age of 70 years gives an age-graded performance of 80%.

The MAs were drawn as a sub-sample from a larger study that included 286 MAs with accelerometry measurements and of those, 189 participants also additionally completed DXA assessments at the Manchester research centre. These 189 participants with both accelerometry and DXA data were included in the present study. The DXA images from two participants were excluded due to movement artefacts, so data are presented from 187 individuals with valid DXA and accelerometry data.

Control participants were individuals recruited as part of the EU "MYOAGE" study [211] using advertisements in newspapers and University of the Third Age with the aim to recruit socially active individuals. Volunteers were excluded if: dependent living, unable to walk a distance of 250m, presence of morbidity (such as neurologic disorders, metabolic diseases, rheumatic diseases, heart failure, severe chronic obstructive pulmonary disease and haemocoagulative syndromes), immobilisation for

one week during the last three months, orthopaedic surgery during the last two years and/or suffering from pain or functional limitations.

3.1.4.2 DXA Scans

Standing height was measured to the nearest millimetre and body mass was measured to the nearest 0.1 kg. Whole body, total hip and lumbar spine dual energy X-ray absorptiometry (DXA) scans were performed using a DXA scanner while the participant lay supine (Lunar Prodigy Advanced, GE Healthcare, encore version 10.50.086). During the measurements, a light cotton t-shirt was worn by the participants to reduce measurement errors due to clothing absorption. Body composition (fat mass and lean mass) was measured from total body scans, whilst bone mineral density (BMD, $g.cm^{-2}$) was measured from hip and spine scans. All measurements were recorded after manual adjustment of the regions of interest carried out offline. Repeat total body and hip DXA scans were performed in 8 MAs within one month of the original scan. Using these repeat scans the short-term error for our laboratory was 2.0% for hip BMD and 0.9% for spine BMD.

3.1.4.3 Muscle function

A Leonardo Mechanography Ground Reaction Force Platform (Leonardo Software version 4.2: Novotiec Medical GmbH, Pforzheim, Germany) was used to assess lower limb muscle function during a vertical jump as described previously [212] and described below. From this, both absolute and relative power was assessed. Briefly, the participants performed a two-footed countermovement jump where each participant was asked to jump as high as they could. Jumps were performed with a trained assistant present and in reach of the participants in case of a fall or falter. Each

participant repeated the jump sequence three times, with approximately 30 seconds rest between jumps. The jump with the maximum power was used for statistical analysis.

3.1.4.4 Accelerometry

Accelerometry data was collected from the athletes only. Each athlete received a GCDC ×16–1c (Gulf Coast Data Concepts, Waveland, Mississippi) which was placed in a Velcro strap and worn around the waist with the accelerometer device placed over their right hip. Each athlete wore this monitor for 7 consecutive days, only removing it when showering, bathing, swimming and sleeping. The monitor was kept on for all other daily activities including athletic training. Time sheets were completed over the 7-day period to identify the time the monitor was first worn, the time it was removed in the evening and to indicate any reason why that day was not of their usual routine. Accelerometers were configured with standardised settings prior to participant use with a sampling frequency of 50 Hz, a deadband setting of 0.1 g (the threshold which must be exceeded before a recording is made) and a timeout setting of 10 s (meaning that a single sample every 10 s is taken even if the recording is <0.1 g) [102]. Once the period of use was completed the participant returned the accelerometer to the centre, by post, where the raw accelerometry data was then uploaded to a secure shared drive and read into Stata 13 (StataCorp, College Station, TX). A standardized cleaning and processing procedure was used [102]. The Y-axis accelerations data were cleaned to remove movement artefacts and any periods of nil data collection, presumably due to the participant not wearing the accelerometer. Activity data were normalised based on seven valid days of 14 hours with ≥10 h recording time. Y-axis peaks were calculated based on accelerations higher than the previous and subsequent reading

and recorded within 14 pre-specified g bands. These were condensed to three impact bands; low (≥0.5 to <1.0 g), medium (≥1.0 to <1.5 g) and higher (≥1.5 g) impact. All g values represent g over and above 1 g from earth's gravitational force [76].

3.1.4.5 Statistical Analysis

Statistical analysis was performed using SPSS for Windows (v21, IBM, USA). Data was firstly assessed for normality of distribution using P-P and Q-Q graphs, and the Kolmogorov-Smirnov test. Accelerometry data was not normally distributed, so this data was log transformed for further analysis. Non-normally distributed data are presented as median (25^{th}/75^{th}) quartiles and all other data are presented as mean ± standard deviation (SD).

Univariate ANOVA analysis with Fisher's Least Significant Difference post-hoc tests was used to identify differences between the three groups (sprinters, endurance runners and controls). Males and females were combined in the statistical analysis and differences were determined with adjustment for sex. There was no evidence of group * sex interaction, therefore data from both sexes were combined for analysis. Differences were considered significant at $p<0.05$. Lean mass [213] and muscle function [214] are highly correlated with bone strength, even when accounting for allometric scaling. Therefore, these and other co-variates were included to assess group differences in bone outcomes using a series of five different models, as shown in Table 3. Model 1: age, height, sex; Model 2: model 1 + fat mass; Model 3: model 1 + lean mass; Model 4: model 1 + lean mass + fat mass; Model 5: model 4 + absolute power.

To further investigate factors associated with bone outcomes in the athletes, single factor linear regression was performed for each individual variable (age, height, AGP, training age, hours trained, fat mass, lean mass, body mass, absolute power, vertical impacts (low, medium and high), discipline and sex) in relation to hip and spine BMD, for the athlete groups combined. Next, a stepwise linear regression was conducted with the athlete groups combined, using the same variables, to determine predictors of hip and spine BMD within Master Athletes. Results of regression analyses are presented as standardised regression coefficients (ß) and 95% confidence interval unless otherwise stated.

3.1.5 Results

3.1.5.1 Participant characterisation

Participant characteristics are shown in Table 1. Controls were older than both sprint and endurance runners. There was no difference between any groups in height. Endurance runners were lighter and had lower BMI than both sprinters and controls, and sprinters also had lower BMI than controls. Controls had 32% and 40% higher body fat percentage than sprinters and endurance runners, respectively. Sprinters had greater lean mass and 10-30% greater relative and absolute power values than both endurance runners and controls. Lean mass but not absolute or relative power was also greater in endurance runners than controls.

Table 1. Participant characteristics separated by group and sex.

Variable	Group						Group pair-wise comparisons		
	1. Sprint		2. Endurance		3. Controls		1 vs 2	1 vs 3	2 vs 3
Sex	M	F	M	F	M	F			
N	28	10	111	38	29	30			
Age (years)	70.9±6.4	71.5± 7.9	69.9±5.7	69.1±50	74.1±5.7	73.3±4.5	.181	**.022**	**<.0005**
Height (cm)	174±6	162±6	173±6	162±7	172±9	160±5	.467	.104	.180
Body mass (kg)	74.3±9.7	62.9±10.9	67.9±7.7	56.0±7.4	80.2±16.2	63.1±11.5	**<.0005**	.098	**<.0005**
BMI ($kg.m^{-2}$)	24.5±2.7	23.9±3.8	22.5±3.1	21.4±2.1	27.1±4.7	24.5±4.2	**<.0005**	**.012**	**<.0005**
Lean mass (kg)	57.8±5.6	43.8±4.7	54.2±5.4	41.3±5.0	52.2±8.4	38.1±4.3	**.001**	**<.0005**	**.007**
Fat (%)	17.3±5.7	25.0±10.3	15.6±5.7	22.5±7.2	29.9±8.7	34.2±9.0	.136	**<.0005**	**<.0005**
Absolute Power (kW)	2.64±0.90	1.68±0.59	2.03±0.56	1.41±0.38	2.19±0.59	1.46±0.49	**<.0005**	**.002**	.179
Relative Power ($W.kg^{-1}$)	35.3±10.4	27.3±11.0	30.1±8.1	25.2±6.0	27.5±5.2	23.0±4.9	**.002**	**<.0005**	.060

Values are mean ± standard deviation; *P*-values for post hoc comparisons between groups are shown after adjusting for sex.

3.1.5.2 Characteristics related to athletic training

Mean age-graded performance ranged was 82.2% across the cohort, indicating a high level of performance as shown in Table 2. Age-graded performance was greater in sprinters than endurance runners. There was no difference in the number of hours per week trained between sprinters and endurance. The number of impacts recorded in the low and medium bands were 2.2- and 3.0-fold higher, respectively, in endurance than sprint athletes, but the number of counts in band 3 (high impacts) did not differ between endurance and sprinters.

Table 2. Athlete-specific characteristics, separated by athletic group and sex.

Variable		Group				Group Difference *P*
		1. Sprint		2. Endurance		
Sex		M	F	M	F	
Training Age (years)	<18	17	5	57	8	.140
	18-29	2	1	12	3	
	30-49	6	1	22	11	
	>50	3	3	18	15	
Current Training Hours per week	0-1	0	0	2	0	0.111
	1-3	5	2	13	4	
	4-7	18	4	59	19	
	7+	4	3	36	15	
Age graded Performance (%)		82.3± 13.6	89.5± 11.4	76.5± 10.7	80.6± 10.2	**.002**
Accelerometry low impact (0.5-1g) counts		20876 (12362-40738)	14368 (6623-33408)	40882 (28228-53412)	37161 (25787-55780)	**<.0005**
Accelerometry medium impact (1-1.5g) counts		6434 (2364-13692)	3326 (694-13081)	33458 (18847-49909)	29868 (21076-41859)	**<.0005**
Accelerometry (counts) high impact (>1.5g) counts		131 (9-693)	37 (4-293)	193 (20-1038)	90 (12-774)	.291

Values are mean ± standard deviation except accelerometry counts (median (IQR)), *P*-values for post hoc comparison between groups are shown after adjusting for sex.

3.1.5.3 Bone Mineral Density

In minimally-adjusted Model 1, mean hip BMD in sprinters was ~10% greater than endurance runners and 9% greater than controls (Table 3). Adjustment for fat mass in Models 2, 4 and 5 increased the differences between sprinters and controls, whilst adjustment for lean mass in Model 3 had little effect on group differences. There were no differences in hip BMD between endurance and controls for any model (all $P > 0.15$).

Sprinters had greater spine BMD than endurance athletes in Model 1 and this remained the case after further adjustment in Models 2, 3, 4 and 5. There was no difference in spine BMD between sprinters and controls in minimally-adjusted model 1 or after lean mass adjustment in Model 3. However, adjustment for fat mass in models 2, 4 and 5 showed values to be higher in sprinters than controls. Conversely, greater spine BMD was found in controls than endurance runners in models 1 and 3, but these group differences were fully attenuated by adjustment for fat mass in models 2, 4 and 5. The adjusted means for each model of adjustment are presented in Figure 3.1 ((A; hip BMD) and (B; spine BMD)).

Table 3. Bone outcomes separated by group and sex.

Variable	Group							Group pair-wise comparison		
	1. Sprint		2. Endurance		3. Controls		Model	1 vs 2	1 vs 3	2 vs 3
	M	F	M	F	M	F				
Hip BMD ($g.cm^{-2}$)	1.15±0.16	0.97±0.11	1.03±0.15	0.88±0.11	1.05±0.12	0.88±0.13	**1**	**<.0005**	**0.006**	0.184
							2	**<.0005**	**0.001**	0.953
							3	**<.0005**	**0.016**	0.159
							4	**<.0005**	**0.002**	0.993
							5	**0.001**	**0.007**	0.980
Spine BMD ($g.cm^{-2}$)	1.21±0.21	1.02±0.18	1.09±0.13	0.89±0.03	1.15±0.17	0.95±0.15	**1**	**<.0005**	0.110	**0.010**
							2	**<.0005**	**0.001**	0.763
							3	**0.002**	0.345	**0.005**
							4	**0.001**	**0.009**	0.957
							5	**0.004**	**0.018**	0.923

Values are mean ± standard deviation, *P*-values for post hoc comparison between groups are shown after adjustment for sex. Adjustments: M1; adjusted for sex, height and age, M2; M1 + fat mass, M3; M1 + lean mass, M4; M1+ fat mass + lean mass, M5; M4+ absolute power

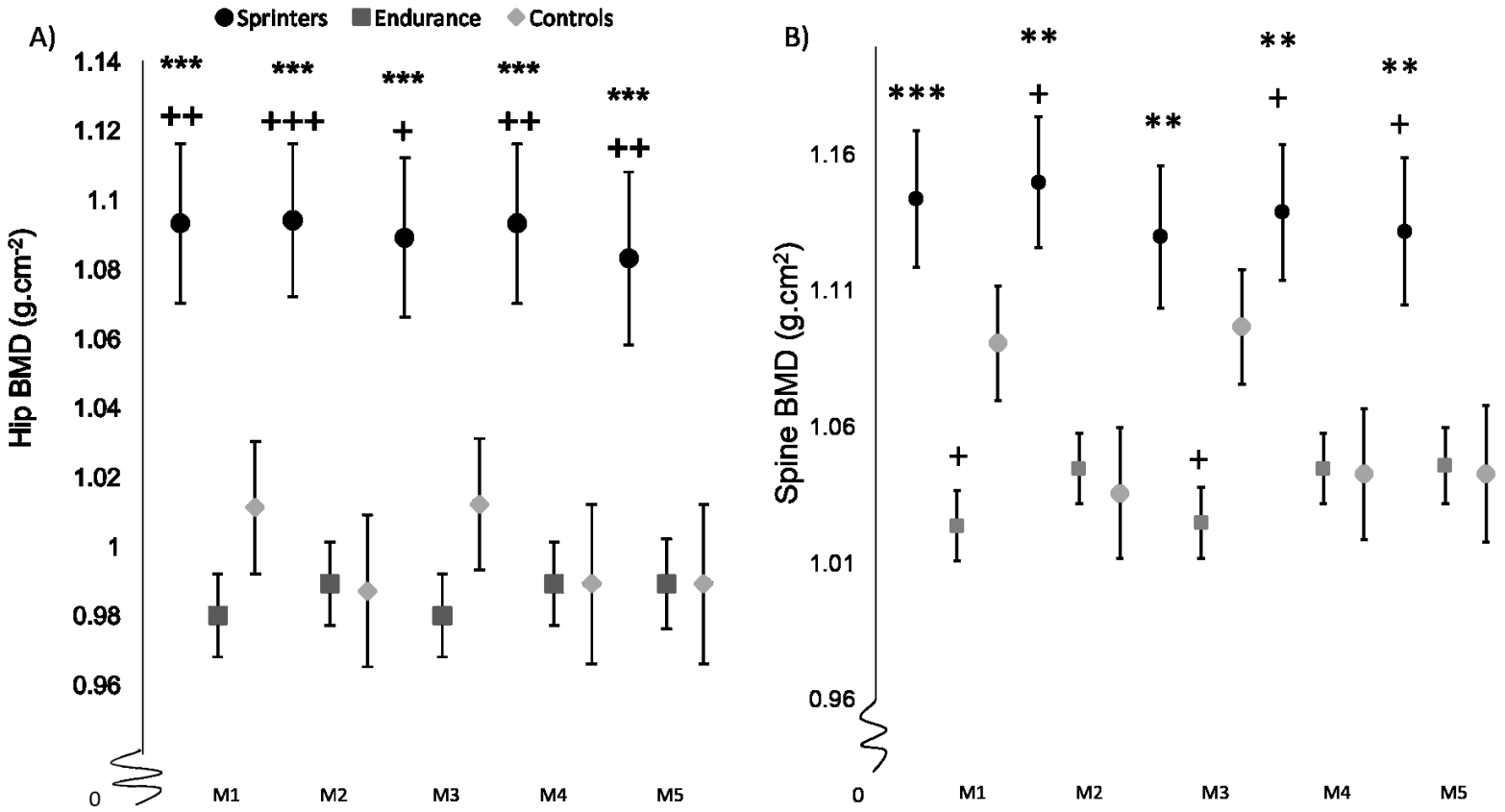

Figure 3.1. Adjusted mean estimates separated by group in a series of ANOVA models (means ±SD) for A) hip and B) spine BMD adjustments: M1; adjusted for sex, height and age, M2; M1 + fat mass, M3; M1 + lean mass, M4; M1+ fat mass + lean mass, M5; M4+ absolute power. Asterisks indicate significant difference from endurance *- $P < 0.05$, * - $P < 0.01$, *** - $P < 0.001$. Crosses indicate significant difference from controls +- $P < 0.05$, ++ - $P < 0.01$, +++ - $P < 0$.

3.1.5.4 Regression Analysis

Results of linear regressions between individual athlete characteristics and bone outcomes, when adjusted for age, height, body mass and sex, are shown in Table 4. Discipline (sprinter), AGP and absolute jump power were positively associated with hip BMD, whilst later starting age, low and medium impact counts were negatively associated with hip BMD. Discipline (sprinter), training age, and fat mass were positively associated with spine BMD, whilst a later starting age, low and medium impacts were negatively associated with spine BMD.

Table 4: Results of linear regression between each individual athlete characteristic and bone outcomes in athletes only

Variable		Hip BMD				Spine BMD			
		β	95% CI		P	β	95% CI		P
Discipline (Sprinter)		0.281	0.155	0.408	**<0.001**	0.268	0.145	0.390	**<0.001**
AGP		0.131	-0.004	0.266	0.059	0.100	-0.029	0.229	0.130
Training Age[1]		-0.231	-0.366	-0.096	**0.001**	-0.153	-0.284	-0.021	**0.024**
Training Hours[1]		0.017	-0.118	0.151	0.809	0.017	-0.109	0.144	0.791
Fat Mass		0.102	-0.032	0.235	0.137	0.230	0.104	0.356	**<0.001**
Lean Mass		0.093	-0.168	0.354	0.485	0.216	-0.039	0.471	0.099
Absolute Jump Power		0.150	-0.005	0.304	0.060	0.174	0.023	0.326	**0.025**
Accelerometry Counts	Low	-0.177	-0.040	-0.314	**0.012**	-0.246	-0.117	-0.375	**<0.001**
	Medium	-0.169	-0.032	-0.306	**0.016**	-0.296	-0.169	-0.422	**<0.001**
	High	0.109	-0.034	0.252	0.137	-0.056	0.080	-0.193	0.420

Data are adjusted for age, height and sex presented as standardised regression coefficient (β). [1]Test for linear trend between categories.

In stepwise multiple linear regressions, the variables identified as predictors of hip BMD were sex (greater values in males, standardised regression coefficient 0.393, 95%CI 0.257 to 0.529, $P < 0.001$), discipline (greater values in sprinters, 0.246, 95%CI 0.113 to 0.38, $P < 0.001$), age (-0.259, 95% CI, -0.128 to -0.39, $P < 0.001$) and starting age (-0.168, 95%CI -0.03 to -0.307, $P = 0.012$). For spine BMD sex (male, 0.527, 95%CI 0.4 to 0.654, $P < 0.001$), discipline (sprinter, 0.248, 95%CI 0.121 to 0.374, $P < 0.001$) and age (-0.13, 95%CI -0.003 to -0.257, $P = 0.046$) were identified as predictors.

3.1.5.5 Sensitivity Analyses

To examine the influence of regional lean mass on bone, analyses adjusted for appendicular or lower limb lean mass rather than whole body measures was performed. In addition, analyses with lean and fat mass indices (lean or fat/height^2 respectively) and relative jump power was also carried out. Results of these alternative analyses (data not shown) were similar to those described above, therefore whole body measures and unadjusted body composition and peak power values were retained in analyses.

3.1.6 Discussion

The main finding was that hip and spine BMD were greater in sprinters than endurance athletes and non-athletic controls. These differences remained after adjustments for body composition and muscle function. Endurance athletes had lower spine BMD than controls during initial analysis, but this difference disappeared after adjusting for body fat. These findings suggest that long-term endurance exercise has little benefit for hip and spine BMD. In contrast, long-term sprint training may help to preserve hip and spine BMD at levels considerably higher than those of non-athletic controls. This is the first study to compare hip and spine BMD of older master athletes from different training disciplines and controls in a large cohort. The hip and spine are important clinically because they are prone to fracture in old age. Previous studies were limited by the absence of a control group [170] or discipline-specific comparisons [206, 215], recruitment of middle-aged athletes [208], or focused on distal or less fracture-prone regions rather than hip and spine [207, 208, 215].

The findings support previous observations of greater BMD in sprinters compared with endurance runners and controls [8, 170, 208] . A previous DXA study in younger master athletes (40-64y) reported similar bone outcomes for endurance athletes and controls, whilst distal tibia trabecular BMD as assessed by pQCT were greater for both sprint and endurance runners compared to controls [8] . The differences between hip and tibia adaptations to different forms of running could be explained by the biomechanics of running at different speeds. Knee and hip torques increase with increasing running speed, but the torque around the ankle tends to plateau at speeds above 5 metres per second [216].

In terms of factors underlying the discipline-specific advantages in hip BMD in sprinters which has been observed here and by others [170, 208, 217], sprinters had higher lean mass and jumping power than endurance runners and controls. Though absolute and relative jumping power was positively associated with both hip and spine BMD, this relationship was no longer observed once discipline was included in the regression, which other than sex and age was the only independent predictor of BMD at both sites. Taken together, these observations suggest that whereas differences in muscle function likely contribute to observed BMD differences between sprinters and endurance runners, this influence is only partially explained by muscle power as measured by jumping mechanography. This limitation may reflect that whilst a number of parameters relevant to bone loading, that were previously shown to be associated with bone outcomes, were measured [6, 76, 214, 218, 219], outcomes were not able to directly assess bone deformation, nor the loads placed upon bones by reaction and muscle forces. A previous study employing detailed biomechanical assessment of running gait in sprint athletes identified kinetic variables as predictors of bone strength within a master sprinter population [220]. More detailed biomechanical analyses within different athletic populations may identify relevant components of the training stimulus. Moreover, BMD is influenced by lifelong exposure to mechanical strain, as indicated by greater hip and spine BMD in retired youth athletes [221] at old age. The muscle measures were only obtained at a single point in time relatively late in life. Given the known decrease in muscle bulk and function with age [211] particularly in athletes [222], this study may have significantly underestimated differences in muscle function between these two groups across the life-course.

Mechanical loading on the skeleton is a reflection not only of muscle function, but also

participation in physical activity. High impact activities, even when rare are thought to be osteogenic based on positive associations found between high vertical impact activity and bone outcomes in non-athletic older individuals [202, 223]. The expectation was that sprinters would achieve greater numbers of high impacts than endurance athletes which was hypothesised to contribute towards their greater BMD. Whereas BMD was substantially higher in sprinters, the endurance athletes and sprinters had similar numbers of high impacts as measured using accelerometry. It should be noted that the accelerometers only registered vertical impacts and not horizontal components of acceleration. Indeed, the power output and, most likely, the magnitude and rate of strains experienced by the bones during sprinting, are greater than those during endurance running predominately due to the horizontal rather than vertical impulses [103]. Further research is needed to test whether overall (horizontal and vertical) accelerations are associated with bone adaptations observed in sprint but not endurance runners.

It is also conceivable that vertical impacts of lower magnitude, in the low and medium range, exert osteogenic activity. However, whereas we have previously observed that master athletes have considerably higher levels of low and medium vertical impact activity compared to controls [224], and in the present study endurance runners showed even greater numbers of low and medium impacts compared to sprinters, BMD in endurance runners was similar to that of controls and below that of sprinters. Indeed, low and medium impacts were inversely related to BMD. This inverse relationship may reflect the recent observation that low and medium impacts as recorded here are inversely related to BMI [225], of which the latter is positively

related to bone mass [226]. Within this study the observation was that spinal BMD was in fact lower than controls in minimally-adjusted models, which differences attenuated after adjustment for fat mass, is consistent with this explanation. The absence of bone benefits in endurance runners could also be related to desensitisation of the bone by regular low-level habitual activity [227], and/or saturation of the response to high-magnitude loading after a very small number of loading cycles [228, 229]. Therefore, the higher levels of low and medium-impact activity performed by endurance than sprint and control athletes may not contribute positively to bone strength.

An alternative explanation to mechanical influences explaining the difference between sprint and endurance athletes' BMD could be a pre-existing self-selection bias in sport participation, possibly relating to aspects of body stature not captured by our methodology but otherwise influencing BMD. This possibility has been proposed in a number of previous master athlete studies [206, 207, 230], but never explored. Studies of bone health in individuals beginning to take part in sprint and endurance events either in childhood or adulthood could examine whether such bias exists.

3.1.6.1 Strength and Limitations

The main strength of this study is the comparison of a large cohort of elite level master athletes competing at very high levels and with extensive training history of different disciplines, and controls. This allowed the impact of muscle strength, body mass, body composition and vertical impacts on the BMD at the hip and spine, sites which are clinically important due to their susceptibility to bone fractures in old age, to be assessed. Previous studies have omitted comparisons with controls, considered younger athletes or did not investigate these fracture-prone regions [170, 207, 208].

However, the cross-sectional nature of the study limits assessment of causal relationships between type of sport and BMD due to possible uncontrolled confounders. For instance, there was little information about other factors potentially related to bone health, such as use of medications and nutrient intake including vitamin D, but it seems unlikely that these will have differed substantially between groups so as to explain the BMD differences we observed. In addition, a detailed training log was not taken, so some additional information about differences in exposure to higher impacts between sprinters and endurance runners may have been missed. Another consideration is displacement of the accelerometer during training in extreme high impacts, affecting accuracy of readings. Additionally, master athletes may represent some selection bias in that they are genetically predisposed to these events, which is why they have achieved their success. These findings do not suggest that sprinting activity will prevent any ageing comorbidities. Further longitudinal studies would be needed to identify if sprinting produces greater increases in BMD compared to endurance running in those that have not been competing for the majority of their lives, before translating these findings to the general population.

3.1.6.2 Conclusions

Master sprint runners have greater BMD at the fracture-prone hip and spine sites, and greater lean mass and muscle power than healthy non-athletic controls, but no such advantages in BMD were evident in endurance runners. BMD advantages in sprinters were only partly explained by differences in lean mass and muscle function, whilst further adjustment for other indicators of skeletal loading including accelerometry measures within sprinters and endurance runners could not explain group differences. Further studies are required to identify to what extent discipline-specific advantages in

BMD relate to pre-existing differences in skeletal health, or to variance in skeletal loading not captured in this study.

4.1 Chapter 4: Circulating levels of Dickkopf-1, Osteoprotegerin and sclerostin are higher in old compared with young men and women and positively associated with whole-body bone mineral density in older adults

4.1.1 Abstract

Purpose: To investigate the relationship between whole-body bone mineral density (WBMD) and levels of circulating factors with known roles in bone remodelling during 'healthy' ageing.

Methods: WBMD and fasting plasma concentrations of dickkopf-1, fibroblast growth factor-23, osteocalcin, osteoprotegerin, osteopontin and sclerostin were measured in 272 older subjects (69 to 81 years; 52% female) and 171 younger subjects (18-30 years; 53% female).

Results: WBMD was lower in old than young. Circulating osteocalcin was lower in old compared with young, while dickkopf-1, osteoprotegerin and sclerostin were higher in old compared with young. These circulating factors were each positively associated with WBMD in the older adults and the relationships remained after adjustment for covariates (r-values ranging from 0.174 to 0.254, all $p<0.01$). In multivariate regression, the body mass index, circulating sclerostin and whole-body lean mass together accounted for 13.8% of the variation with WBMD in the older adults. In young adults, dickkopf-1 and body mass index together accounted for 7.7% of variation in WBMD.

Conclusion: Circulating levels of dickkopf-1, osteocalcin, osteoprotegerin and sclerostin are positively associated with WBMD in community-dwelling older adults, despite the average WBMD being lower and circulating dickkopf-1, osteoprotegerin and sclerostin being higher in old than young.

4.1.2 Introduction

The findings from Chapter 3 have shown that sprinters have an increased bone mineral density at the hip and spine, compared to endurance master athletes and controls. The differences could not be explained by the number of impacts, nor muscle function. Given this, the differences could possibly be accounted for by the interaction of circulating factors with bone. The following chapter investigates the correlation between circulating factors and bone mineral density in young and old populations, addressing the second objective for the PhD.

Progressive loss of bone mineral density (BMD) in older age leads to osteoporosis as the balance of bone remodelling favours resorption of mineralised extracellular matrix over formation. This common change is characterized by 'micro-architectural' deterioration of bone tissue and increases the risk of fracture [2]. BMD can be affected by BMI, muscle size or the sex of the individual. Males generally have a higher BMD than females and a lower incidence of osteoporosis in older age [231]. The greater muscle size will induce a greater muscle force on the bone when contracting [4, 232-234] and may stimulate osteoblasts at a greater rate, resulting in higher BMD [4, 234].

Circulating factors influencing bone development have been implicated in the age-related changes to BMD including interactions between 1,25 dihydroxyvitamin D_3 (25(OH) VitD), parathyroid hormone (PTH) and calcium [235-238]. In addition, regulatory factors released from osteoblasts during bone formation and osteoclasts during bone resorption can enter the circulation and their concentrations may be related to BMD in older age.

There are many circulating factors that could be involved in bone and muscle cross talk. Due to the bone-muscle interactions it may be feasible that the circulating factors could be associated with BMD and/or lean mass, but research in this area is lacking. Some of the candidate circulating factors possibly related to BMD include osteoprotegerin (OPG), which is expressed by osteocytes and osteoblasts and can reduce production of osteoclasts by binding receptor activator of nuclear factor kappa-B ligand (RANKL) [11]. Osteocalcin (OC) is a major non-collagen protein of the bone matrix secreted by osteoblasts for bone formation, but released from the matrix during bone resorption [239]. Dickkopf-1 (DKK1) [14] and sclerostin, released primarily by osteocytes [15], negatively regulate bone formation and have emerged as therapeutic targets to tackle osteoporosis [186]. Fibroblast growth factor 23 (FGF23) is produced by a variety of cell types, including osteoblasts and osteocytes, and released into the circulation where it acts on the kidney to increase excretion of phosphate and reduce production of 1-25 OH Vitamin D [187]. Osteopontin (OPN) is an extracellular matrix protein released by osteoblasts, osteocytes and osteoclasts to facilitate bone resorption [16].

It remains unclear how the combination of these circulating markers of bone turnover are related to BMD in older age. Therefore, the purpose of this study was to compare plasma concentrations of these markers between recreationally active, community dwelling older adults and a reference group of young adults, and to examine the association of these with whole-body bone mineral density (WBMD). It was hypothesised that older adults would have higher circulating levels of factors related to bone resorption compared with young, and higher circulating markers of bone resorption were expected to be associated with lower BMD in old age.

Therefore, the purpose of this part of the study (addressing Objective 2) was to measure plasma concentrations of selected factors with known regulatory roles in bone remodelling; to compare their concentrations between recreationally active, community dwelling older adults and a reference group of young adults, and to examine the association of these with whole-body bone mineral density (WBMD).

4.1.3 Materials and Methods

4.1.3.1 Study Design

The cross-sectional European multi-centre MYOAGE cohort consists of relatively healthy older men and women (aged 69 to 81 years) and young adults (aged 18-30 years) [211] as shown in table 5. The study was approved by ethics committees at each institute and written informed consent was obtained from all participants. Participants were recruited by advertisement in newspapers, the University of the Third Age and Association of Emerti. All measurements were performed according to standard operating procedures that had been unified at the study centres and data collection was ceased through December-March and July-August. Volunteers were excluded if: dependent living, unable to walk a distance of 250 m, presence of morbidity (such as neurologic disorders, metabolic diseases, rheumatic diseases, heart failure, severe chronic obstructive pulmonary disease and hemocoagulative syndromes), immobilization for one week during the last three months and orthopaedic surgery during the last two years or still causing pain or functional limitations. The inclusion and exclusion criteria were designed to ensure the selection of relatively healthy participants and to minimize the confounding effect of comorbidity on sarcopenia [211] and the use of bisphosphonates, calcium and vitamin D supplements was recorded. The present study included 443 participants (Leiden, The Netherlands

(young; n=35, old; n=75); Jyvaskyla, Finland (young; n=34, old; n=65); Tartu, Estonia (young; n=39, old; n=60), Paris, France (young; n=35, old; n=30) and Manchester, UK (young; n=28, old; n=42)) with complete BMD and bloods results.

4.1.3.2 Dual-energy X-ray absorptiometry

A whole body scan was performed using DXA while the participants lay supine, as previously reported [211] (The Netherlands: Hologic QDR 4500, version 12.4, Hologic Inc., Bedford, MA, USA; Finland: Lunar Prodigy, version en-Core 9.30; Estonia: Lunar Prodigy Advanced, version en-Core 10.51.006; France: Lunar Prodigy, version encore 12.30; United Kingdom: Lunar Prodigy Advance, version enCore 10.50.086). A trained technician completed the daily equipment calibration and the DXA scans according to local and manufacturers' quality control procedures. Participants wore a light cotton garment to reduce effects of clothing absorption on the scanning results. The whole-body lean mass, fat mass and the WBMD were recorded after manual adjustment of the regions of interest carried out after the scan was complete.

4.1.3.3 Blood sample analysis

Blood samples were collected from a vein in the forearm into vacutainer EDTA tubes in the morning when participants were in a fasted state. Samples remained at room temperature for 15-30 min and were then centrifuged for 15 min at 2,000 *g at 4°C. The plasma was collected and stored at -80°C until analysis. Plasma concentrations of the selected analytes were determined in the research laboratory in Manchester, UK, using multiplex immunoassays (Millipore, Billerica, MA, USA). The manufacturer instructions were followed and the magnetic bead panels quantified DKK1, OPG, OC, OPN, sclerostin and FGF23 using a 96-well plate after an overnight incubation. The

sensitivity of each analyte was 1.4 (DKK1), 1.9 (OPG), 68.5 (OC), 37.7 (OPN), 31.1 (sclerostin) and 9.2 (FGF23) pg/mL. Samples were processed using a Luminex 200 Bioanalyser and protein concentrations were estimated using the xPONENT software (Luminex, v.3.1.871).

4.1.3.4 Statistical analysis

Participant descriptive characteristics (Table 5) were normally distributed and are presented as mean ± standard error of the mean (SEM). Comparisons between age and gender were assessed using multivariate ANOVA. Relationships between body stature, BMI, total body lean mass and supplement use (independent variables) with WBMD (dependent variable) were assessed using bivariate Pearson's product moment correlation. Data for circulating factors were not normally distributed and are presented as median (25^{th}/75^{th}) centiles. The results were log-transformed and z-scores calculated by expressing each log-transformed value as a standard deviation from the mean of the gender-matched young. Z-scores of WBMD, lean mass and BMI were also calculated for use in subsequent correlation and regression analysis. Spearman's rho partial correlations were performed to assess relationships between the z-score WBMD with z-scores of circulating factors using two models. The first model included adjustment for country of testing to account for any systematic differences. The second accounted for the positive correlations we observed between WBMD and BMI in men and women (r-values ranging from 0.210 – 0.387) and WBMD and lean mass for men (r-values in men ranging from 0.268 – 0.357, and women 0.085 – 0.099) as well as health status and use of bisphosphonates, calcium or vitamin D supplements. Thus, the second model included adjustments for: country of testing, z-score of lean mass, z-score of BMI, self-reported health and supplement use. A

stepwise multiple linear regression using the self-reported health and supplement use as well as z-scores for BMI, lean mass and circulating factors was then used to evaluate which combination of the independent variables was associated with z-score WBMD (dependent variable) in older adults and in young adults. Data was analysed using SPSS for Windows (v.21; IBM, USA) and significance accepted as $p<0.05$.

4.1.4 Results

Based on z-scores relative to gender-matched young, 26% of the older participants had WBMD values between -1.5 to -2.49 below the mean for young and 10.6% were ≥ -2.5 below the mean of young. There was a significant age-by-gender interaction for WBMD z-scores ($p<0.0005$).

There were gender differences in all participant characteristics except for age (Table 1). WBMD was significantly lower in old compared with young participants. Total lean mass was lower in old compared with young when expressed in absolute values (Kg) and also as percentage of total body mass due to old having higher fat mass than young. There was a significant age by gender interaction for total lean mass ($p=0.025$) and for WBMD z-scores ($p<0.0005$).

Table 5. Participant descriptive characteristics

	Old		Young		p-value	
	Men (n=129)	Women (n=143)	Men (n=82)	Women (n=89)	Age	Gender
Age (years)	74.6±0.3	74.0±0.3	23.6±0.3	23.2±0.3	**<.0005**	NS
Height (m)	1.74±0.01	1.61±0.01	1.81±0.01	1.67±0.01	**<.0005**	**<.0005**
Body mass (kg)	78.8±1.0	65.1±0.8	75.4±1.2	62.4±1.0	**<.0005**	**.018**
BMI (kg/m^2)	25.8±0.3	25.2±0.3	23.1±0.3	22.4±0.3	**.017**	**<.0005**
Body fat (kg)	20.1±0.7	22.7±0.6	12.9±0.7	18.8±0.7	**<.0005**	**<.0005**
Lean mass (kg)	55.9±0.6	40.2±0.5	59.9±0.9	41.4±0.6	**<.0005**	**<.0005**
Body fat (%)	25.5±0.6	34.6±0.6	16.6±0.7	29.6±0.7	**<.0005**	**<.0005**
Lean mass (%)	71.9±0.6	63.0±0.6	79.8±0.7	67.2±0.7	**<.0005**	**<.0005**
WBMD (g/cm^2)	1.19±0.01	1.04±0.01	1.25±0.01	1.15±0.01	**<.0005**	**.001**
WBMD (z-score)	-0.63± 0.10	-1.47± 0.11	0.00±0.11	0.00±0.11	**<.0005**	**<.0005**

Values are mean ± SEM. WBMD: whole-body bone mineral density. NS; no significant difference.

In older men and women, WBMD was significantly associated with body mass (r=0.407, p<0.0005; r=0.241, p=0.004, respectively), but not height (r=0.160, p=0.072; r=-0.087, p=0.308, respectively). In young men, WBMD was significantly associated with body mass and height (r=0.386, p<0.0005; r=0.218, p=0.050, respectively) and in young women WBMD was significantly associated with body mass, but not height (r=0.218, p=0.041; r=0.070, p=0.518, respectively). WBMD was significantly associated with BMI in older men (r=0.387, p<0.0005), older women (r=0.316, p<0.0005), younger men (r=0.329, p=0.003) and younger women (r=0.210; p=0.050). WBMD was also associated with lean mass in older men (r=0.268, p=0.002) and younger men (r=0.357,

p=0.001), but not in older women (r=-0.085, p=0.322) or younger women (r=0.099, p=0.361).

Table 6 shows concentrations of the circulating factors. Compared with young, older participants had higher concentrations of DKK1, OPG and sclerostin. Concentrations of OC were significantly lower in old compared with young. OPN and FGF23 did not differ significantly between young and older participants although this was after removal of 37% of FGF23 samples [similar proportions of young and old] that fell below the level of assay detection. Compared with men, women had higher circulating concentrations of OPG, but lower OPN and sclerostin. There were no significant differences between men and women for DKK1, FGF23 and OC. Age x gender interactions were found for OC, OPG and sclerostin (all $p<0.05$): the difference between young and old in OC, OPG and sclerostin was greater for men than it was for women.

Table 7 shows the associations between circulating bone regulatory factors and WBMD. When using z-scores of all variables and including all participants, while adjusting for country, WBMD was positively associated with DKK1. This association remained significant after additionally adjusting for lean mass, BMI, self-reported health and supplement use. In older participants only, DKK1, OC, OPG and sclerostin were positively associated with WBMD after adjusting for country. This remained the case when additionally adjusting for lean mass, BMI, self-reported health and supplement use. In younger participants only, DKK1 was positively associated with WBMD after adjusting for country as well as when additionally adjusting for lean mass, BMI, self-reported health and supplement use.

Table 6. Circulating markers of bone remodelling in old and young, men and women.

	Old		Young		p-value		
	Men	Women	Men	Women	Age	Gender	Age x Gender
DKK1 ($pg.mL^{-1}$)	577.0 ± 352-804	575.3 ± 346-864	420.6± 290-627	494.3 ± 284-703	**<.0005**	.942	.843
FGF23 ($pg.mL^{-1}$)	113.5 ± 72-274 (n=75)	103.0 ± 64-211 (n=87)	122.9.7 ± 87-195 (n=54)	141.7 ± 94-225 (n=60)	.792	.316	.700
OC ($pg.mL^{-1}$)	14160.5 ± 9911-18708	16065.4 ± 11073-19933	17581.1 ± 13304-21223	16733.9 ± 12013-20715	**<.0005**	.880	**.036**
OPG ($pg.mL^{-1}$)	319.2 ± 229-419	306.9 ± 257-392	159.4 ± 114-193	208.5 ± 160-260	**<.0005**	**<.0005**	**<.0005**
OPN ($pg.mL^{-1}$)	26590.1 ± 17094-38028	21350.1 ± 13971-31255	24822.5 ± 16928-35662	20877.5 ± 15937-27777	**.**700	**.009**	.184
Sclerostin ($pg.mL^{-1}$)	5690.3 ± 4348-7556	4147.6 ± 3349-5159	3016.1 ± 2079-3932	2366.0 ± 1923-3134	**<.0005**	**<.0005**	**.034**

Values are median ± 25^{th} – 75^{th} percentiles. For FGF23, the *n* is less than those given in Table 1 due to some samples having values that were below the level of detection. The *n* for all other analytes is the same as shown in Table 1.

Table 7. Associations between circulating bone regulatory factors and whole body bone mineral density.

Correlation with z-score WBMD	all participants combined		Old		Young	
Adjustment models	1	2	1	2	1	2
DKK1	**r=.107 p=.026**	**r=.129 p=.008**	**r=.167 p=.007**	**r=.174 p=.005**	**r=.263 p=.001**	**r=.282 p<.0005**
FGF-23	r=.067 p=.274	r=.051 p=.406	r=-.095 p=.235	r=-.079 p=.330	r=-.086 p=.370	r=-.130 p=.182
OC	**r=-.124 p=.010**	r=-.083 p=.088	**r=.150 p=.015**	**r=.187 p=.003**	r=-.023 p=.767	r=-.008 p=.916
OPG	**r=-.096 p=.047**	r=-.039 p=.419	**r=.209 p=.001**	**r=.254 p<.0005**	r=.081 p=.297	r=.055 p=.484
OPN	r=-.005 p=.918	r=-.001 p=.980	r=.055 p=.370	r=.073 p=.245	r=-.120 p=.124	r=-.122 p=.120
Sclerostin	r=-.091 p=.059	r=-.075 p=.126	**r=.241 p<.0005**	**r=.240 p<.0005**	r=.129 p=.096	r=.135 p=.086

Data are shown as spearman's rho. The circulating bone regulatory factors were log-transformed and their z-scores calculated. The p value indicates the level of significance after statistical analysis. Results were adjusted for 1) country; 2) country, z-score lean mass, z-score BMI, self-reported health and supplement use. Significant relationships are highlighted using bold text.

Stepwise multiple linear regression was performed including z-score WBMD as the dependent variable and independent variables included: self-reported health, supplement use and z-scores of the variables BMI and lean mass, as well as the z-scores derived from log-transformed data for DKK1, FGF23, OC, OPG, OPN and sclerostin. Results in the young showed DKK1 accounted for 5.1% of the variation in WBMD (adjusted r^2=0.051, p=0.010), while DKK1 and BMI accounted for 7.7% of the variation in WBMD (adjusted r^2=0.077, p=0.005). In the old, BMI alone accounted for 8.9% of the variation in WBMD (adjusted r^2=0.089, p<0.0005); BMI and sclerostin together accounted for 12.0% of the variation in WBMD (adjusted r^2=0.120, p<0.0005), while BMI, sclerostin and whole body lean mass accounted for 13.8% of the variation in WBMD (adjusted r^2=0.138, p<0.0005).

4.1.5 Discussion

The results of this study showed that circulating factors DKK1, OPG and sclerostin were each higher in old compared with young, but positively associated with WBMD in older adults. Circulating OC was lower in old compared with young and positively associated with WBMD. In multivariate regression, BMI, circulating sclerostin and whole-body lean mass together accounted for 13.8% of the variation with WBMD in the older adults. In young, DKK1 and BMI together accounted for 7.7% of variation in WBMD.

Low BMI [240] and low lean mass [3] in older age are known risk factors for osteopenia and osteoporosis, possibly due to lower loading on bones, particularly on weight-bearing bones. Female gender and low BMI were independently associated with the lowest quartile (within the sample) of WBMD, while male gender, higher lean mass and BMI were independently associated with the highest quartile for WBMD. These findings were consistent with a previous report [161] and with other research linking age-related declines in BMD to reduced mechanical strains [241].

4.1.5.1 Circulating factors associated with whole-body BMD

Four out of the six circulating factors differed in concentration between old and young (Table 2). Of those, DKK1, OC, OPG and sclerostin were identified from both partial correlation models as associated with WBMD in older participants (Table 3). Sclerostin and DKK1 are released primarily by osteocytes and inhibit bone formation by blocking the osteoblast Wnt/β-canenin signalling pathway [14, 242], with sclerostin and DKK1 also stimulating bone resorption through RANKL [243]. Down-regulation of sclerostin [186] and DKK1 [14, 186] is associated with markedly increased bone formation. For these reasons, an inverse association between circulating sclerostin and

DKK1 with WBMD would be expected, but is not entirely what was observed. In line with expectations, these results revealed, on average, a 1.8 fold higher circulating sclerostin and approximately 1.2-fold higher DKK1 in old compared with young, which is consistent with an inverse association between sclerostin and BMD in older age [244] and with results from a small sample of 36 patients showing an inverse association between DKK1 and lumbar and femur BMD [245]. However, contrary to expectations, the circulating levels of sclerostin and DKK1 were positively associated with WBMD in the older participants (Table 3). Similar positive associations between circulating sclerostin with BMD and bone micro-architecture in old age has been previously reported [246-249].

Similar to the findings for sclerostin and DKK1, a paradoxical relationship existed for OPG and WBMD in older adults: circulating OPG was higher in old compared with young (Table 2), but circulating OPG was positively associated with WBMD (Table 3). OPG released by osteocytes and osteoblasts promotes bone formation. It has been shown to protect against generalised bone resorption by blocking TNFα in models of chronic inflammation [250] and is considered to be a decoy receptor for RANKL to reduce osteoclast-driven bone resorption [251]. There are conflicting reports about the direction of association between circulating OPG and BMD. A study of postmenopausal women of mean age 62 years [252], and a study of middle aged men [253] reported inverse relationships between BMD and OPG, while others reported no relationship [254, 255]. Conversely, and in line with the results of the present work, when adults in their eighth and ninth decades of life were included in the sample population the relationship between OPG and BMD was positive [256, 257]. These conflicting results cannot be explained by the differences between studies in skeletal

site examined. Conflicting results may be related to the differences in the age range of the study samples and possible gender differences. The results for OPG and sclerostin showed significant age x gender interactions indicating that the differences between young and older men were greater than those between young and older women (Table 2). It is already known that sex hormones can regulate bone turnover and may interact with these circulating factors [258].

It is not clear why circulating sclerostin, DKK1 and OPG were positively associated with WBMD in older age, despite the conflicting overall trend for higher circulating levels and lower WBMD in the old. One possibility is that the older, but healthy mature osteocytes generally release higher absolute levels of sclerostin, DKK1 and OPG into the circulation [259] [260]. For example, a positive correlation was found for circulating sclerostin with trabecular density, number and thickness in older men [246, 259], suggesting the more advanced trabecular resorption in osteoporotic bone leaves fewer mature osteocytes and thus, lower sclerostin release than healthy older bone. However, analysis of bone biopsies showed similar sclerostin mRNA levels in young and old despite higher circulating sclerostin levels in the old [244] which indicates that the age-related differences in circulating sclerostin may not be due to increased osteocyte sclerostin gene expression, although this does not necessarily equal protein production [261].

Lower circulating OC was found in old compared with young (Table 2) and, consistent with this, circulating OC levels were positively correlated with WBMD in the old (Table 3). OC released by osteoblasts plays a role in bone formation, so the positive correlation with WBMD may be expected. However, others suggest that higher

circulating OC indicates greater rates of bone resorption because fragments or whole OC protein is released into the circulation during bone resorption [239]. A previous study of young and middle-aged women suggested that circulating levels peaked soon after menopause and dropped thereafter, although levels were higher in those with osteoporosis than those without [262]. Interestingly, these results also show a positive association between DKK1 and WBMD in the young adults from univariate and multivariate analyses. This association may be a reflection of the numbers of mature osteocytes or related to total bone mass, but more work is needed to confirm. One previous study of children and adolescents did not find any association between circulating DKK1 and BMD, but the young included in that study of youths were in stages of rapid developmental growth, which could present different results from the steady- state of young adults [263].

4.1.5.2 Strengths and limitations

The MYOAGE study included young and older participants relatively free from lifestyle-related comorbidities for their age and the results are therefore indicative of age-related effects. Nevertheless, the associations identified in this cross-sectional study cannot be interpreted as causal relationships despite the clear roles for the selected circulating markers in bone remodelling. The results for FGF23 showed no significant age- or gender-differences, nor correlations with WBMD, but a large proportion of the results were below the level of assay sensitivity, so firm conclusions cannot be drawn for this analyte. Circulating levels of markers have been measured, which may be influenced by release from non-bone cells, so it is not possible to determine the originating cell type. It is possible that altered renal function can affect the levels of the circulating factors, but markers of renal function was not included in the present

study due to limitation of plasma sample quantity. A phantom was not used to calibrate the DXA scanners across sites and results were not adjusted to derive "standardised" DXA values, as others have done for hip and femur sites [264]. Instead, all study centres followed the local quality control procedures, including use of phantoms and daily calibration and the results were adjusted for country of testing to account for possible systematic differences.

Future studies should determine the reasons for the positive relationship between circulating sclerostin, DKK1 and OPG with BMD in older adults, despite the old having on average higher circulating levels of these factors and lower WBMD.

4.1.5.3 Conclusion

Sclerostin, DKK1, OPG and OC were each positively associated with WBMD in older adults, despite the average WBMD being lower and circulating DKK1, OPG and sclerostin being higher in old than young. Multiple linear regression identified BMI, circulating sclerostin and whole-body lean mass as explaining approximately 14% of all variation on WBMD amongst older adults.

5.1 Chapter 5: Circulating markers associated with whole body lean mass in older men and women.

5.1.1 Abstract

Purpose: To investigate the relationship between whole-body lean mass (WB Lean mass) and levels of circulating factors with known roles in bone remodelling during 'healthy' ageing.

Methods: WB Lean mass and fasting plasma concentrations of dickkopf-1 (DKK1), osteocalcin (OC), osteoprotegerin (OPG), osteopontin (OPN), sclerostin and tumour necrosis factor-alpha (TNFα) were measured in 272 older subjects (69 to 81 years; 52% female) and 171 younger subjects (18-30 years; 53% female). Spearman's rho partial correlations were performed to assess associations between the z-score WB Lean mass with z-scores of circulating factors. Multivariate regression was used to examine the relationships between lean mass and the circulating factors.

Results: WB Lean mass was 5% lower in old than young. Circulating osteocalcin was lower in old compared with young, while dickkopf-1, osteoprotegerin, sclerostin and TNFα were higher in old compared with young. These circulating factors were each significantly ($p<0.03$) negatively associated with WB Lean mass in the older adults (OC; r=-0.175, DKK1; r=-0.312, OPG; r=-0.170, Sclerostin; r=-0.235) apart from TNFα that was positively associated (r=0.140) with WB Lean mass in older adults. In multivariate regression, height and OPG accounted for 45% of the WB Lean mass in the older adults. In young the same combination of OPG and height accounted for 18% of variation in WBMD.

Conclusion: These results identify OPG as a circulating factor significantly associated with whole body lean mass in young and older adults.

5.1.2 Introduction

Chapter 4 has shown that circulating factors Osteoprotegrin (OPG), Sclerostin, Dickkopf-1 (DKK1) and Osteocalcin (OC) are significantly associated with whole body bone mineral density in older adults. Fourteen percent of the variation in whole body bone mineral density can be explained by BMI, sclerostin and whole body lean mass. Muscle and bone interactions have been highlighted in section 2.1.7. It is possible that the same circulating factors associated with whole body bone mineral density are also associated with whole body lean mass. This chapter, addresses objective 3, and investigates the associations between circulating factors, as measured in Chapter 4, and whole body lean mass in the old and young population.

Skeletal muscle mass decreases progressively with advancing older age towards a condition known as sarcopenia characterized by low appendicular muscle mass, associated weakness and reduced physical function [115, 135, 265]. At the same life stage, the skeletal bone mineral density (BMD) also declines, progressing through stages of “reduced” BMD, known as osteopenia, and “low” BMD known as osteoporosis [44, 59, 68]. Low muscle mass and low bone mineral density share similar risk factors, including older age, low activity levels and changed hormone profiles [136, 138].

Although it is possible to have low muscle mass without low BMD in older age and vice-versa, these conditions more often co-occur [266-269]. Direct interactions may link the changes affecting muscles with those of bone as muscle forces are transmitted to bones during physical activity to provide a growth stimulus. This regulation of muscle on bone is commonly known as the ‘mechanostat theory’ [4, 82, 88, 174],

explaining how high muscle mass is associated with higher BMD [83, 270] while, conversely, low muscle mass is associated with lower BMD [98, 271]. However, direct responses to loading are not the only possible mechanism of interaction between muscle and bones, since both respond to growth factors in the local milieu. Factors released by muscle or bone tissue might regulate growth or catabolic processes of the other tissue, evidencing bi-directional interactions and building substantially upon the mechanostat theory, which describes only effects of muscle on bone.

Circulating factors have been associated with BMD amongst relatively healthy older men and women [272]. For instance, concentrations of DKK1, OPG and Sclerostin were higher in old than young adults, while OC was lower in old compared with young. DKK1, Sclerostin, OC and OPG were positively associated with BMD in older age [272]. These factors released by cells, including osteocytes, can regulate osteoblasts or osteoclasts during bone formation and resorption, but may also act on skeletal muscles and be associated with muscle mass in older age. For instance, Osteoclacin has been shown to influence muscle and bone cells [13, 189]. Diminishing Osteocalcin by gene knock-out or receptor knock-out leads to low exercise capacity [13, 190] and this is also the case when osteocalcin knock-out is restricted to osteoblasts [189] highlighting the importance of the bone-muscle cross-talk. Bone morphometric proteins (BMPs) are also involved in bone and muscle adaptations. BMPs are involved in limb bone patterning during development [273] but also have been shown to inhibit the myogenic process of muscle development, with chick limb bud cultures, and possibly transform the myoblasts to a chondrogenic fate, eventually forming cartilage [274]. Using mouse models, it has been shown the BMP-2 can induce bone formation whilst also up-regulating BMP receptors on muscle fibres causing muscle regeneration

[275]. OPG has also been shown to interact with muscle, in particular OPG can regenerate muscle force within fast twitch fibres in muscle degenerative diseases, such as muscular dystrophy, using mice models. It also acts a regulator in vascular smooth muscle cells, acting as a down regulator of vascular calcification [276-279]. These studies demonstrate that muscle tissue responds to factors usually associated with bones. However, little is currently known about their association with muscle mass or changes with ageing in humans.

The purpose of the present study was to investigate whether the selected circulating factors previously associated with bone, are related to whole body lean mass of young and older men and women.

5.1.3 Materials and Methods

5.1.3.1 Study Design

The cross-sectional European multi-centre MYOAGE cohort consists of relatively healthy older men and women (aged 69 to 81 years) and young adults (aged 18-30 years) [211] as shown in Table 8. The study was approved by ethics committees within each institute and written informed consent was obtained from all participants. Participants were recruited by advertisement in newspapers, the University of the Third Age and Association of Emerti. All measurements were performed according to standard operating procedures that had been unified at the study centres and data collection was ceased through December-March and July-August. Volunteers were excluded if: dependent living, unable to walk a distance of 250 m, presence of morbidity (such as neurologic disorders, metabolic diseases, rheumatic diseases, heart failure, severe chronic obstructive pulmonary disease and hemocoagulative syndromes), immobilization for one week during the last three months and

orthopaedic surgery during the last two years or still causing pain or functional limitations. The inclusion and exclusion criteria were designed to ensure the selection of relatively healthy participants and to minimize the confounding effect of comorbidity on sarcopenia [211] and we recorded the use of bisphosphonates, calcium and vitamin D supplements. The present study included 443 participants (Leiden, The Netherlands (young; n=35, old; n=75); Jyvaskyla, Finland (young; n=34, old; n=65); Tartu, Estonia (young; n=39, old; n=60), Paris, France (young; n=35, old; n=30) and Manchester, UK (young; n=28, old; n=42)) with complete BMD and bloods results.

5.1.3.2 Dual-energy X-ray absorptiometry

A whole body scan was performed using DXA while the participants lay supine, as previously reported [211] (The Netherlands: Hologic QDR 4500, version 12.4, Hologic Inc., Bedford, MA, USA; Finland: Lunar Prodigy, version en-Core 9.30; Estonia: Lunar Prodigy Advanced, version en-Core 10.51.006; France: Lunar Prodigy, version encore 12.30; United Kingdom: Lunar Prodigy Advance, version enCore 10.50.086). A trained technician completed the daily equipment calibration and the DXA scans according to local and manufacturers' quality control procedures. Participants wore a light cotton garment to reduce effects of clothing absorption on the scanning results. The whole-body lean mass, fat mass and the WBMD were recorded after manual adjustment of the regions of interest carried out after the scan was complete.

5.1.3.3 Blood sample analysis

Blood samples were collected from a vein in the forearm into vacutainer EDTA tubes in the morning when participants were in a fasted state. Samples remained at room temperature for 15-30 min and were then centrifuged for 15 min at 2,000 *g at 4° C.

The plasma was collected and stored at -80°C until analysis. Plasma concentrations of the selected analytes were determined in the research laboratory in Manchester, UK, using multiplex immunoassays (Millipore, Billerica, MA, USA). The manufacturer instructions were followed and the magnetic bead panels quantified DKK1, OPG, OC, OPN and sclerostin using a 96-well plate after an overnight incubation. The sensitivity of each analyte was 1.4 (Dicckopf-1; DKK1), 1.9 (Osteoprotegrin; OPG), 68.5 (Osteocalcin; OC), 37.7 (Osteopontin; OPN) and 31.1 (sclerostin) pg/mL. Samples were processed using a Luminex 200 Bioanalyser and protein concentrations were estimated using the xPONENT software (Luminex, v.3.1.871). TNFα was sent to an external lab for analysis.

5.1.3.4 Statistical analysis

Participant descriptive characteristics (Table 1) were normally distributed, as assessed by Kolmogorov-Smirnov test, and are presented as mean ± standard error of the mean (SEM). Comparisons between age and gender were assessed using multivariate ANOVA. Relationships between height, whole body bone mineral density (WBMD), total body fat %, self-reported health and supplement use (independent variables) with whole body (WB) Lean mass (dependent variable) were first assessed with univariate correlations to identify the independent predictors of WB Lean mass. Then using bivariate Pearson's product moment correlations were identified between the independent variables and WB Lean mass. Data for circulating factors were calculated as Z-scores per country as a standard deviation from the mean of the gender-matched young. Z-scores of WBMD, fat mass and height were also calculated for use in subsequent correlation and regression analysis. Spearman's rho partial correlations were performed to assess relationships between the z-score WB Lean mass with z-

scores of circulating factors using two models. The first model included adjustment for WBMD and height (accounting for the link between osteocytes and the circulating factors). The second model additionally accounted for differences that may be seen within the use of bisphosphonates, calcium or vitamin D supplements and self-reported health as well as differences that may occur in height and WB Fat %. A stepwise multiple linear regression using the self-reported health and supplement use as well as z-scores for height, WB Fat %, WBMD and circulating factors was then used to evaluate which combination of the independent variables was associated with z-score WB lean mass (dependent variable) in older adults and in young adults. Data was analysed using SPSS for Windows (v.21; IBM, USA) and significance accepted as $p<0.05$

5.1.4 Results

Table 8. Participant Descriptive Characteristics

	Old		Young		p-value	
	Men (n=129)	Women (n=143)	Men (n=82)	Women (n=89)	Age	Gender
Age (years)	74.6±0.3	74.0±0.3	23.6±0.3	23.2±0.3	**<.0005**	NS
Height (m)	1.74±0.01	1.61±0.01	1.81±0.01	1.67±0.01	**<.0005**	**<.0005**
Body mass (kg)	78.8±1.0	65.1±0.8	75.4±1.2	62.4±1.0	**<.0005**	**.018**
BMI (kg/m^2)	25.8±0.3	25.2±0.3	23.1±0.3	22.4±0.3	**.017**	**<.0005**
Body fat (kg)	20.1±0.7	22.7±0.6	12.9±0.7	18.8±0.7	**<.0005**	**<.0005**
Lean mass (kg)	55.9±0.6	40.2±0.5	59.9±0.9	41.4±0.6	**<.0005**	**<.0005**
Body fat (%)	25.5±0.6	34.6±0.6	16.6±0.7	29.6±0.7	**<.0005**	**<.0005**
Lean mass (%)	71.9±0.6	63.0±0.6	79.8±0.7	67.2±0.7	**<.0005**	**<.0005**
WBMD (g/cm^2)	1.19±0.01	1.04±0.01	1.25±0.01	1.15±0.01	**<.0005**	**.001**

Values are mean ± SEM. WBMD: whole-body bone mineral density. NS: No significant difference.

Table 8 shows participant characteristics. Compared with young, the older adults were shorter, with higher total body and fat mass, but lower lean mass and WBMD. Compared with men, women were shorter, with lower total body and lean mass, lower WBMD, but higher body fat percentage. There was a significant age * gender interaction for WBMD, ($p<0.0005$), but no age*gender interactions for any other characteristics.

To identify associations, univariate correlations were assessed between BMI, supplement use, self-reported health, height, total body fat percentage and WBMD, all

with WB Lean mass as the dependent variable, were significantly correlated with WB Lean mass (BMI; r=0.347 p<.0005, Supplement use; r=-0.278 p<.0005, WBMD r=0.520 p<.0005, Fat % r=-0.522 p<.0005, Self-reported health r=-0.164 p=0.008, Height r=0.868 p<.0005). For young, all were significantly correlated except for supplement use and self-reported health (BMI; r=0.416 p<.0005, Supplement use; r=0.065 p=0.402, WBMD; r=0.557 p<.0005, Fat %; r=-0.611 p<.0005, Self-reported health; r= -0.077 p=0.322, Height; r=0.840 p<.0005). Based on these significances for old and young, all variables were therefore used as covariates subsequent multiple regression models.

Table 9. The concentration of selected circulating factors in plasma in old and young, men and women.

	Old		Young		p-value		
	Men	Women	Men	Women	Age	Gender	Age x Gender
DKK1 ($pg.mL^{-1}$)	577.0 ± 352-804	575.3 ± 346-864	420.6± 290-627	494.3 ± 284-703	**<0.0005**	.942	.843
OC ($pg.mL^{-1}$)	14160.5 ± 9911-18708	16065.4 ± 11073-19933	17581.1 ± 13304-21223	16733.9 ± 12013-20715	**<0.0005**	.880	**.036**
OPG ($pg.mL^{-1}$)	319.2 ± 229-419	306.9 ± 257-392	159.4 ± 114-193	208.5 ± 160-260	**<0.0005**	**<0.0005**	**<0.0005**
OPN ($pg.mL^{-1}$)	26590.1 ± 17094-38028	21350.1 ± 13971-31255	24822.5 ± 16928-35662	20877.5 ± 15937-27777	.700	**.009**	.184
Sclerostin ($pg.mL^{-1}$)	5690.3 ± 4348-7556	4147.6 ± 3349-5159	3016.1 ± 2079-3932	2366.0 ± 1923-3134	**<0.0005**	**<0.0005**	**0.034**
TNFα ($pg.mL^{-1}$)	1.79 ± 1.53-2.09	1.65 ± 1.31-2.11	1.47 ± 1.10-1.77	1.51 ± 1.18-1.68	<**0.0005**	.156	.242

Values are median ± 25^{th} – 75^{th} percentiles.

As described in the previous chapter, compared with young, older participants had higher concentrations of DKK1, OPG and sclerostin. Concentrations of OC were

significantly lower in old compared with young. OPN did not differ significantly between young and older participants. Compared with men, women had higher circulating concentrations of OPG, but lower OPN and sclerostin. There were no significant differences between men and women for DKK1 and OC. Age x gender interactions were found for OC, OPG and sclerostin (all $p<0.05$): the difference between young and old in OC, OPG and sclerostin was greater for men than it was for women.

Table 10. Circulating factors associations with whole body lean mass, in old and young, men and women.

Correlation With Z score WB lean mass	All participants combined		Old		Young	
Adjusted models	1	2	1	2	1	2
DKK1 ($pg.mL^{-1}$)	r=-0.099 p= 0.601	**r=-0.247 p=<.0005**	**r=-0.191 p=0.002**	**r=-0.312 p=<.0005**	r=0.017 p=0.832	r=-0.130 p=0.098
OC ($pg.mL^{-1}$)	r=-0.209 p=0.267	**r=-0.183 p=<.0005**	r=0.029 p=0.649	**r=-0.175 p=0.005**	r=0.022 p=0.779	r=-0.179 p=0.023
OPG ($pg.mL^{-1}$)	r=-0.059 p=0.756	**r=-0.083 p=<.0005**	**r=-0.163 p=0.009**	**r=-0.170 p=0.007**	r=-0.076 p=0.337	**r=-0.204 p=0.009**
OPN ($pg.mL^{-1}$)	**r=-0.371 p=0.043**	**r=-0.111 p=0.023**	r=-0.021 p=0.735	**r=-0.144 p=0.022**	r=-0.111 p=0.162	r=-0.147 p=0.062
Sclerostin ($pg.mL^{-1}$)	r=-0.004 p=0.982	**r=-0.152 p=0.002**	**r=-0.266 p=<.0005**	**r=-0.235 p=<.0005**	r=0.009 p=0.908	**r=-0.248 p=0.001**
TNFα ($pg.mL^{-1}$)	r=0.130 p=0.495	**r=0.229 p=<.0005**	r=-0.103 p=0.113	**r=0.140 p=0.030**	r=-0.079 p=0.343	**r=-0.173 p=0.037**

Data are shown as spearman's rho. The circulating factors were log-transformed and then their z-scores calculated per country, compared to young values. The p values indicate the level of significance after statistical analysis. Results were adjusted for 1) Z WBMD and height; and 2) model 1 +Z fat %, self-reported health and supplement use. Significant relationships are highlighted using bold text.

Table 10 shows the associations between circulating bone regulatory factors and WB lean mass. When using z-scores of all variables and including all participants, while adjusting for WBMD and height (model 1), WB Lean mass was negatively associated only with OPN. All factors examined were negatively associated with WB Lean mass when additionally adjusting for WB fat %, self-reported health and supplement use (model 2), except for TNFα, which was positively associated.

Negative associations between WB Lean mass and DKK1, OPG and Sclerostin were significant when restricting analyses to older participants for model 1. For model 2, all factors were negatively associated with WB lean mass in old, except for TNFα, which showed a positive association. When analysis was restricted to young participants, there were no significant associations for circulating factors and WB lean mass using model 1. However, in model 2, OPG, Sclerostin and TNFα were each negatively associated with WB lean mass (Table 10).

Stepwise multiple linear regression was performed including Z-score WB Lean mass as the dependent variable and independent variables including: self-reported health and supplement use as well as the Z-scores for DKK1, OC, OPG, OPN and sclerostin, WB Fat %, WBMD and height. Results in the young showed OPG accounted for 12.8% of the variation in WB Lean mass (adjusted r^2=0.118, p<0.0005), while OPG and height accounted for 18.6% of the variation in WB Lean mass (adjusted r^2=0.186, p<0.0005). OPG, height and, WB Fat% accounted for 25.3% of the variation in WB Lean mass (adjusted r^2=0.253, p<0.0005). OPG, height, WB Fat%, and DKK1 accounted for 28.9% of the variation in WB Lean mass (adjusted r^2=0.289, p<0.0005).

In the old, height alone accounted for 33.7% of the variation in WB Lean mass (adjusted r^2=0.337, p<0.0005); height and OPG together accounted for 45.2% of the variation in WB Lean mass (adjusted r^2=0.452, p<0.0005), while height, OPG and DKK1 accounted for 49.4% of the variation in WB Lean mass (adjusted r^2=0.494, p<0.0005). Height, OPG, DKK1 and WB Fat% accounted for 50.8% of the variation in WB Lean mass (adjusted r^2=0.508, p<0.0005). Height, OPG, DKK1, WB Fat% and OC accounted for 51.7% of variation in WB Lean mass (adjusted r^2=0.517, p<0.0005).

These results consistently identify OPG as associated with WB Lean mass, both in young and in older adults

5.1.5 Discussion

Whole body muscle mass declines markedly with advancing older age, progressing towards sarcopenia characterized by low muscle mass and physical function. The results of the present study suggest higher circulating levels of DKK1, OPG, OPN and sclerostin, and lower circulating levels of OC, are associated with lower whole body lean mass in older adults after considering body composition and stature. The association with OPG was most consistent, accounting for almost 12% of variation in whole-body lean mass of older adults and almost 13% for younger adults in multivariate analysis.

OPG is a member of the tumor necrosis factor (TNF) super family, along with RANK (receptor activated nuclear-Kb) and RANKL (Ligand for RANK). This RANK/RANKL/OPG signalling pathway is a key regulator of vascular calcification [276] and bone formation/resorption [280, 281]. OPG is a soluble decoy receptor and can bind to RANKL preventing activation by RANK. The discovery of this pathway contributed to

the development of a bisphosphonate known as denosumab, that uses RANKL antagonists to halt the activity of osteoclasts and osteoblasts to help preserve bone mineral density [282], but actions may extend beyond bone, since OPG is also secreted by C2C12 skeletal myoblasts in culture [279], possibly to serve autocrine or paracrine functions.

The present study is the first to associate circulating OPG levels with lean mass amongst young and older human adults, but there are several other indications of OPG effects on muscle using animal models and cell cultures. RANK regulates calcium storage, sarco-endoplasmic reticulum calcium ATP-ase (SERCA) activity as well as function of fast twitch muscle fibres, [278]. RANK is found on skeletal muscle cells and osteoclasts. Once RANKL binds it is able to activate Calcium signalling pathways as well as affecting the differentiation, activation and survival of osteoblasts. RANK deletion is able to protect from loss of specific muscle force, but not mass, in denervation. OPG immunoglobulin fragment complex (OPG-Fc) injections fully restored functional capacity of the extensor digitorum muscle in muscular dystrophy-induced mouse models [279].

As a member of the TNF super family, it is possible that OPG interacts with TNFα. TNFα is a pro-inflammatory cytokine and used to mark a chronic, low-grade systemic inflammation occurring in a range of conditions, including ageing where it was termed "inflamm-ageing" [283-285] associated with tissue damage [285]. TNFα induces RANKL expression, activating the RANK signalling cascade and causing inflammation or cell apoptosis [286] usually in response to injury. The results of Table 9 show altered circulating levels of both TNFα and OPG in older age and Table 10 shows both to be

independently associated with whole body lean mass. Given both TNFα and OPG are associated with WB Lean mass, possible interactions between these two factors may also occur, influencing lean mass. Others have reported TNFα and OPG interactions [277-279], but none have previously demonstrated the associations with whole-body lean mass at the human level. When considered alongside previous research [272] these results identify OPG as a circulating factor released by bone and muscle cells with the potential to regulate muscle and bone function.

In addition to the relationships for OPG and TNFα discussed above, whole-body lean mass was inversely related to DKK1, OC, OPN and sclerostin during univariate analyses. However, only the relationship between DKK1 and lean mass remained significant after multivariate analysis. This is the first report, based on current literature, of associations between DKK1 and lean mass in older age. DKK1 has previously been shown to act as an antagonist regulating fibrosis formation after injury [287]. Some exercise studies have shown that circulating DKK1 levels decrease over the intervention period whilst muscular strength increases [288], an inverse relationship similar to these findings. Other studies have shown breakdown of muscle during ultra-marathons as well as a decline in DKK1, resulting in less inhibition of osteoblast function [289]. DKK1 has also been shown the play a role in the upregulation of the calcification of vascular smooth muscle cells, in cell cultures exposed to high glucose, replicating diabetic muscle [290]. This highlights possible effects of DKK1 for muscle cells, identifying it as another candidate coordinating muscle-bone cross talk.

5.1.5.1 Strengths and limitations

The strengths and limitations of this study are the same as those that are highlighted in Chapter 4, Section 4.1.5.2. Briefly, they include the cross-sectional study design which cannot reveal causal relationships; the study of relatively healthy participants limiting the findings to only those without mobility limitations of greater burden of disease. Another limitation of this work is that the measurement of circulating regulatory factors means that it was not possible to identify the originating cells releasing the factors into the circulation. Future studies should explore the inter relationship between OPG and TNFα and their respective effects on skeletal muscle cells.

5.1.5.2 Conclusion

Sclerostin, DKK1, OPG and OC were inversely associated with WB Lean mass in older adults. The average WB Lean mass was lower in old than young and the circulating DKK1, OPG and sclerostin were higher in old than young. Multiple linear regression identified, in the old, Height, OPG, DKK1, WB Fat% and OC accounted for 51.7% of variation in WB Lean mass (adjusted r^2=0.517, $p<0.0005$). Of these, OPG was most consistently associated with WB Lean mass.

6.1 Chapter 6: Human skeletal myoblast responses to tumour necrosis factor alpha exposure and the potential protective effects of osteoprotegrin.

6.1.1 Abstract

Purpose; The pro-inflammatory cytokine TNFα causes myoblast cell apoptosis *in vitro*. The present study was designed to investigate the potential protective effects of OPG against TNFα exposure.

Methods; Human myoblast cells were cultured with TNFα alone, OPG alone and both molecules combined. Myoblast differentiation parameters including aspect ratio, fusion index (%) and myotube area (%) were calculated. OPG concentration was measured in cell supernatants and selected 'myokines' were measured from supernatants of myoblasts exposed to TNFα.

Results; Differentiation parameters showed a significantly greater proportion of differentiation when OPG was added to the cultured myoblasts before TNFα ($p<0.0001$). ELISA showed significant secretion of OPG released from human myoblast cells, particularly during the first 24h of incubation ($p=0.001$), with an interaction effect found between TNFα and time F;(15,24) 3.340, $p=0.004$. Other myokines; Osteonectin, Interleukin-6 (IL-6), Leukemia inhibitory factor (LIF) and Fatty acid binding protein (FABP) were also secreted from human myoblast cells as identified using multiplex. Interaction effects were seen for all four myokines; Osteonectin; F (15,24) 7.45, $p<.0005$. LIF; F (15,24) 15.7, $p<.0005$. FABP3; (15,24) 14.5, $p<.0005$. IL-6 F (15,24) 14.5, $p<.0005$.

Conclusion; OPG may protect myoblasts against TNFα exposure and may therefore be used as a therapeutic target for intervention against inflammation associated with ageing.

6.1.2 Introduction

Chapter 5 has identified Sclerostin, Dickkopf-1, Osteoprotegrin and Osteocalcin to be associated with WB Lean mass, the same factors that were identified to be associated with whole body bone mineral density, as highlighted in Chapter 4. More specifically, Chapter 5 has shown Osteoprotegrin to be a consistent factor associated with whole body lean mass, having possible interactions with another circulating factor; tumor-necrosis factor-alpha. Chapter 6 investigates this possible interaction effect, further, using human myoblast cells in culture, addressing the final objective.

Previous research conducted [272], amongst others, has shown that loading (exercise) and molecules released from muscle and bone cells into the circulation can influence both BMD and muscle tissues [181, 291-293]. OPG and TNFα were the two main factors found to be associated with whole body lean mass using our statistical models described in Chapter 5. The previous chapter also highlighted the interactions between OPG and TNFα, by using one another as covariates within the association models. The association between TNFα and lean mass was attenuated after accounting for OPG, but the relationship between OPG and lean mass remained after accounting for TNFα.

OPG is a circulating factor associated with bone formation. RANKL is the receptor activator of NF-κB ligand, RANK is the receptor activator of NF-κB (RANK) and OPG is the soluble decoy receptor of RANKL. RANK, RANKL and OPG are all part of the tumor necrosis factor superfamily [294, 295]. RANKL is expressed on osteoblasts which can then bind RANK causing the formation of osteoclasts, initiating bone resorption. OPG is a decoy receptor for RANKL. This can prevent binding of RANK to its ligand, slowing down the process of bone resorption [296]. The proportion of RANKL to OPG must remain relatively balanced to maintain bone turnover [297]. There have been studies

to show OPG is associated with muscle using C2C12 cells *in vitro*. They have the ability to secrete OPG and an OPG-immunoglobulin fragment complex is able to reverse the effects of muscular dystrophy in mice [277-279]. This pathway also regulates the growth of vascular smooth muscle cells, as the OPG gene suppresses the calcification of these smooth muscle cells [276].

Ageing also triggers a pro inflammatory response characterised by higher levels of circulating TNFα which is linked with a gradual decline in physical activity, presenting as low grade chronic inflammation [144]. Studies have shown that sarcopenia is accompanied by increased levels of inflammation factors, including TNFα and IL-6, and these factors have a catabolic effect over the long-term, accelerating muscle and bone tissue declines [134].

TNFα has been shown to have detrimental effects on myoblast cells in vitro [17, 298-301]. Using C2C12 muscle cell lines, incubation with TNFα results in a total muscle protein loss. Specifically the TNFα binds to its targeted DNA sequence and causes degradation of I-κBα and NF-κB, inducing skeletal muscle protein loss [300]. Furthermore, diaphragm muscles excised from mice overexpressing TNFα have a 47% decrease in contractibility compared to that of control models. It is thought that this contractile dysfunction is a result of endocrine mediated oxidative stress [298]. Some human testing has identified TNFα to be expressed when staining cardiac myocytes, of patients with cardiomyopathy. TNFα is positively correlated with left ventricular volume and negatively correlated with left ventricular systolic function [301].

The primary aim for this chapter was to identify if OPG protects against the effects of TNFα on human myoblasts in vitro. The hypothesis was (1) that OPG will be secreted from myoblasts cultured with TNFα, and (2) OPG protects myoblasts from apoptotic effects of TNFα. There were three main objectives.

1. Identify the optimum concentration of TNFα and OPG to be used in culture with human myoblast cells
2. Identify differences in differentiation parameters when culturing myoblasts with OPG and TNFα, when adding the factors to culture at different time points
3. Using an ELISA and a multiplex kit, identify if human myoblasts secrete OPG and other myokines in response to culturing with TNFα.

6.1.3 Materials and Methods

6.1.3.1 Cell Culture

One 25 year old (C25) non-commercial, immortalized Skeletal muscle cell-line was generated by using transduction with both telomerase-expressing and cyclin-dependent kinase 4-expressing vectors [302]. This cell line was donated to the research group by previous collaborating partners at the Institute of Myology, Paris. A frozen vial containing 1 ml of $1x10^6$ cells each suspended in 90% fetal bovine serum and 10% dimethyl sulfoxide was thawed and transferred into a conical tube of 9 ml prepared complete growth media (GM) for proliferation (Table 1).

Growth Media (GM) Components (as per 500ml)	Concentration
Dulbecco's Modified Eagles Media (DMEM) from Lonza (Nottingham, UK)	60% (320ml)
Medium 199 WITH Earle's BSS from Lonza (Nottingham, UK)	20% (80ml)
Heat inactivated fetal bovine serum (FBS) from Gibco (Loughborough, UK)	20% (100ml)
L-glutamine from Lonza (Nottingham, UK)	1% (5ml)
Fetuin from fetal bovine serum from Sigma-Aldrich (Dorset, UK)	25ug.ml^{-1}
Recombinant human fibroblast growth factor-basic (FGFb) from Gibco (Loughborough, UK)	0.5ng.ml^{-1}
Recombinant human epidermal growth factor (EGF) from Gibco (Loughborough, UK)	5ng.ml^{-1}
Recombinant human hepatocyte growth factor (HGF) from Sino Biological Inc. (Suffolk, UK)	2.5ug,ml^{-1}
Recombinant human insulin from Sigma-Aldrich (Dorset, UK)	5ug.ml^{-1}
Dexamethasone from Sigma-Aldrich (Dorset, UK)	0.2ug.ml^{-1}
Penstrip	10ug.ml^{-1}
Plasmocin	10ng.ml^{-1}

Table 11: Complete Growth Media for Skeletal Muscle cell proliferation.

The 10 ml suspension of the 25-year-old cells (C25) was transferred into a T75 flask. The flask was incubated at 37°C with a 5% CO_2 atmosphere until cell density reached 80% confluence (percentage of area covered in an average field of view). When the flask was 80% confluent, GM (prepared as shown in Table 1) was aspirated from the flask and the cells washed twice with Dulbecco's Phosphate Buffered Saline (DPBS) from Lonza (Nottingham, UK). The cells were then disassociated using 2 ml of TrypLETM Express Enzyme from Gibco (Loughborough, UK) incubated at 37°C in 5%

CO_2 for 5 minutes. The 2 ml cell suspension was transferred into a conical tube and homogenized with 8 ml of GM. The cells were counted on a haemocytometer using 20 µl of cell suspension and 20 µl of Trypan Blue Stain (0.4%) from Lonza (Nottingham, UK). Viable cells.ml^{-1} were calculated using the formula: Average number of live cells in one large corner square x dilution factor x 10^4.

6.1.3.2 Objective 1

Optimisation for the concentration of OPG and TNFα

C25 skeletal muscle cells were seeded at a concentration of 1.5x10^5 cell.ml^{-1} in GM in 6-well plates pre- coated with a 0.5% gelatin solution. Cells were incubated for 24 hours. Subsequently, GM was aspirated and the cells washed twice with DPBS. Differentiation media (DM; Table 2) was added to each well (2 ml) along with a concentration of OPG or TNFα at 5, 10, 15, 20 and 30 ng.ml^{-1}. The plates were then incubated for 48 and 72h. Immunofluorescence microscopy was used to compare the differentiation parameters of both cell lines at 48 and 72h with different concentrations of OPG and TNFα (See Figure 1). At each time point DM was aspirated and cells washed twice with DPBS. Cells were fixed using 4% paraformaldehyde, incubated for 8 minutes at 21°C. Cells were washed thrice with DPBS then permeabilized using 1x Tris (0.5M) TBS containing (0.2% Triton X-100) for 30 min at room temperature. Cells were then blocked using 1x Tris (0.5M) and TBS containing (5% normal goat serum) for 60 mins, at room temperature. Then washed x3 with TBS. Perm/wash buffer was aspirated and the cell washed a final time with DPBS. Cells were then stained with 5 µg.ml^{-1} Texas Red®-X Phalloidin from Invitrogen (Paisley, UK), 5 µg.ml^{-1} Anti-Myosin Heavy Chain Alexa Fluor® 488 from eBioscience (Hatfield, UK), and

2 ng.ml^{-1} 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) from Sigma-Aldrich® (Dorset, UK). The stained cells were visualized using a The Leica DMI6000 B inverted microscope from Leica Microsystems (Milton Keynes, UK), for fluorescent microscopy. ImageJ was used to measure the differentiation parameters. Five random fields of view were assessed at 20x magnification.

Differentiation Media (DM) components as per 500ml	**Concentration**
DMEM (glucose 4.5g.ml^{-1} &L-glutamate) from Lonza (Nottingham, UK)	500ml (100%)
Recombinant Human insulin from Sigma-Aldrich (Dorset, UK)	10μg.ml^{-1}
L-glutamine from Lonza (Nottingham, UK)	1% (5ml)
Penstrip	10μg.ml^{-1} (5ml)
Plasmocin	10ng.ml^{-1}

Table 12: Complete Differentiation Media for Skeletal Muscle cell proliferation.

6.1.3.4 Statistical Analysis for Objective 1

Data was normally distributed as assessed using the Kolmogrov-Smirnov test. For objective 1, a repeated measures ANOVA was used to test whether the two incubation periods (48 and 72 hours) had the same myotube area percentage at the different concentrations (0, 5, 10, 15, 20 and 25ng.ml^{-1} of OPG, and 0, 5, 10, 15, 20 and 30 ng.ml^{-1} for TNFα. All comparisons were made using SPPS v21. Significance was accepted at the $p<0.05$ level. Results are displayed in figure 1.

6.1.3.5 Objective 2

Differentiation Parameters

To achieve objective 2, three six-well plates were seeded with cells as described in section 6.1.3.1. The plates were incubated for 60h with TNFα and OPG as shown in the schematic below.

Plate 1;

TNFα at 0h, OPG at 24h	TNFα at 0h	GM
TNFα at 0h, OPG at 24h	TNFα at 0h, OPG at 24h	DM

Plate 2;

OPG at 0h, TNFα at 24h	OPG at 0h	GM
OPG at 0h, TNFα at 24h	OPG at 0h, TNFα at 24h	DM

Plate 3;

OPG and TNFα at 0h	OPG and TNFα at 0h	GM
OPG and TNFα at 0h	OPG and TNFα at 0h	DM

6.1.3.6 Statistical analysis for objective 2

For this objective 2, a one way ANOVA, with LSD as a post Hoc test, was used to determine the difference between the mean the differentiation parameters, myotube area (%), fusion index (%) and the aspect ratio, at a pre-determined end-point of 60h incubation, between the different co-cultures described above. Myotube area represents the percentage of myotubes within the field of view, the fusion index represents the percentage of nuclei within the myotube compared to the percentage of nuceli within the whole field of view and the aspect ratio represents the ratio of the

length of myotube to the width of the myotube (i.e. the smaller the ratio the higher the level of differentiation). The level of significance was determined at $p<0.05$. Results are shown in Table 13 and Figure 6.2.

6.1.3.7 Objective 3

Co-culture with TNFα for ELISA and multiplex

Human myoblast cells were seeded and cultured as previously stated. When adding DM to the wells of each plate, TNFα was added at a concentration of 30 $ng.ml^{-1}$, as used following on from optimisation. Leaving one well as DM only. Two plates were replicated and the incubated for 24, 48, 72 and 96 hours. The supernatant of each well was collected, labelled and then stored at -80°C until analysis was to be carried out.

6.1.3.8 Concentration of OPG in human cell culture.

The concentration of OPG was measured using an ab100617-Osteoprotegrin Human ELISA kit (Abcam, UK) and was carried out according to the manufacturer's instructions. Reagents and standards were prepped according to the recommendations. 100µl of each standard and sample was added to each well. The plate was then covered and incubated over night at 4 °C with gentle shaking. The solution was then discarded and washed with wash solution four times. 100µL of Biotinylated Osteoprotegrin Detection antibody was added to each well, which was then incubated for 1 hour at room temperature, with gentle shaking. This solution was then discarded, and the plate washed 4 times with wash solution. 100µL of the HRP-Streptavidin solution was added to each well, the plate was then covered and incubated at room temperature for 45 mins with gentle shaking. The solution was

discarded, and the plate washed four times. Finally, 100μL of TMB one-step substrate reagent was added to each well, the plate was then covered and incubated for 30 mins at room temperature, with gentle shaking. Immediately after incubation 50μL of stop solution was added to each well. The plate was then read at 450nm.

The absorbance for standards, controls and samples was calculated. The standard curve was plotted with standard concentration on the x-axis and absorbance on the y-axis. The best-fit straight line was drawn through the standard points and the using the regression equation for the best-fit straight line the concentration of OPG was calculated in $pg.ml^{-1}$.

6.1.3.9 Concentration of myokines in Human cell culture

The concentration of the myokines were determined using a Multiplex immunoassay bead panel (Millipore, Billerica, MA, USA). The manufacturer instructions were followed and the magnetic bead panels quantified; Apelin, Fractalkine, BDNF, EPO, IL-15, Myostatin, Irisin, FSTL, Oncostatin, FGF-21, Osteocrin, Osteonectin, LIF, FABP3 and IL-6, using a 96-well plate after an overnight incubation. The sensitivity of each analyte was $38ng.ml^{-1}$ (Osteonectin), $3pg.ml^{-1}$ (LIF), $6.5pg.ml^{-1}$ (FABP3), $0.6pg.ml^{-1}$ (IL-6), $36pg.ml^{-1}$ (Apelin), $26\ pg.ml^{-1}$ (Fractalkine), $3\ pg.ml^{-1}$ (BDNF), $378\ pg.ml^{-1}$ (EPO), $2\ pg.ml^{-1}$ (IL-15), $163\ pg.ml^{-1}$ (Myostatin), $191\ pg.ml^{-1}$ (Irisin), $548\ pg.ml^{-1}$ (FSTL), $2\ pg.ml^{-1}$ (Oncostatin), $5\ pg.ml^{-1}$ (FGF-21) and $44\ pg.ml^{-1}$ (Osteocrin). Samples were processed using a Luminex 200 Bioanalyser and protein concentrations were estimated using the xPONENT software (Luminex, v.3.1.871). Apelin, Fractalkine, BDNF, EPO, IL-15, Myostatin, Irisin, FSTL, Oncostatin, FGF-21, Osteocrin were all below the level of detection and were not included in further analysis.

6.1.3.10 Statistical Analysis for Objective 3

The third objective was to measure the expression of OPG and other myokines from cultured myoblasts after exposure to TNFα. Univariate ANOVA was performed with time point and concentration of TNFα as fixed factors and OPG concentration as the dependent variable. An interaction effect was found between TNFα*Time, F;(15,24)3.3.40, p=0.004. Given this, simple main effects of the concentration of TNFα (0 or 30ng.ml^{-1}) with the concentration of OPG found within the supernatant were assessed per time point (24, 48, 72 and 96 hours). The effects of TNFα concentration at each time point were evaluated with pairwise comparisons and LSD adjustments. Analysis was performed using SPSS Version 21 (SPSS, Chicago, IL) software and $p<0.05$ was considered statistically significant.

6.1.4 Results

6.1.4.1 Objective1

To investigate the actions of OPG human myoblast cell cultures were performed along with TNFα, a known cytokine that can be inhibited by OPG as a decoy receptor.

To ensure optimal cell differentiation for studies using human recombinant OPG and TNFα, different concentrations (0, 5, 10, 15, 20, 25 ng.ml^{-1} for OPG and 0, 5, 10, 15, 20, 30 ng.ml^{-1} for TNFα) were used concurrently in a 6 well plate lined with 0.5% gelatin. Staining procedure (as described in materials and methods) was carried out at either 48h or 72h. Phase contrast microscopy was used to assess a view at x20 magnification and ImageJ software was used to process images and calculate myotube area percentage for each concentration at the two different time points. Results can be seen in Figure 6.1.

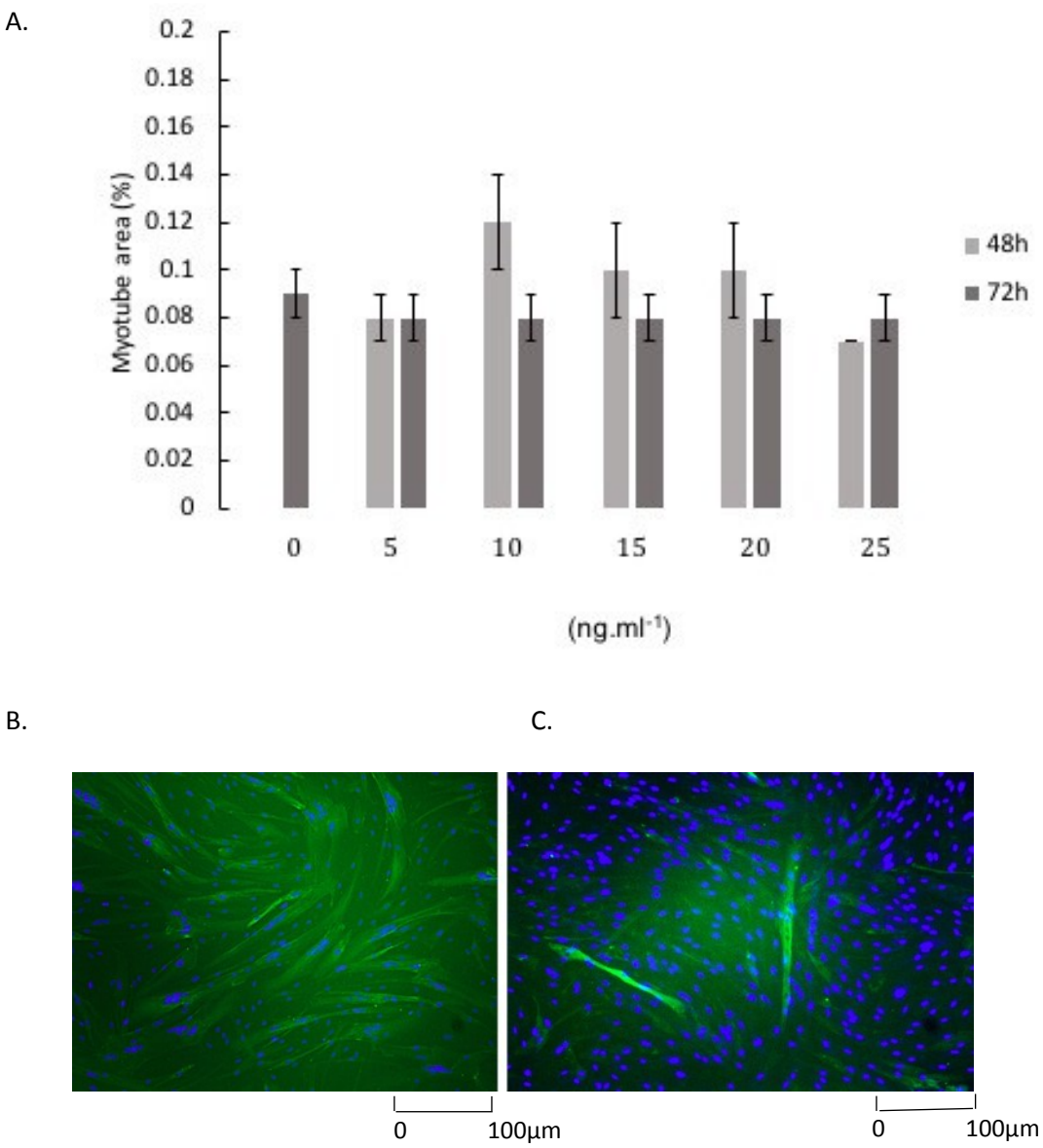

Figure 6.1. A. Determining myoblast percentage with different concentrations of OPG. Myoblast area was calculated with 6 different concentrations of OPG after 48h and 72h of incubation. Data are mean±SEM taken from one x10 magnification image within the specified concentration well. B. Image taken at 72h incubation with 20ng.ml^{-1} of OPG. C. Image taken at 48h incubation with 20ng.ml^{-1} of OPG.

Repeated measures ANOVA was performed to identify any significant differences between times and concentrations. No differences or interactions were found for TNFα or OPG at any of the different time points. When incubating with TNF α alone all myoblast cells died off. Given these results we chose to use 20ng.ml^{-1} for OPG [303, 304] and 30ng.ml^{-1} for TNFα [17, 305] based on previous literature.

6.1.4.2. Objective2

Differentiation parameters highlighted significant differences between the co-cultures (Table 13 and Figure 6.2). Aspect Ratio; OPG and TNFα at 24h was significantly lower (greater differentiation) than TNFα and OPG at 24h and TNFα and OPG at the same time. There was no significant difference in aspect ratio between OPG and TNFα at 24h, TNFα and OPG at 24h and TNFα and OPG together, albeit values were higher for culturing TNFα alone (Table 13). OPG and TNFα at 24h showed a significantly greater fusion index (%), and myotube area (%) than TNFα and OPG at 24h, TNFα and OPG and TNFα only ($p<0.0005$). There was no significant difference in fusion index or myotube area between TNFα and OPG at 24h and, either, TNFα and OPG or TNFα. There was also no significant difference between TNFα and OPG or TNFα only for fusion index or myotube area.

Table 13; Differentiation parameters for different co-cultures

Co-Cultures	Aspect Ratio	Fusion Index (%)	Myotube area (%)
TNFα_OPG at 24h[1]	16.5±2.06[$]	8.85±0.66	0.74±0.07[+"]
OPG_TNFα at 24h[2]	7.70±0.41[*+$]	77.5±3.81[*]	39.9±5.15[*#]
TNFα and OPG[3]	15.1±1.90[$]	6.15±0.66[+]	3.41±0.37[+]
TNFα [4]	10.9±5.45[+]	10.5±0.31[+]	1.01±0.13
OPG[5]	8.72±0.33[*#~]	68.2±0.50[*#~]	32.7±0.73[#]
GM[6]	10.8±2.05[+$]	3.84±0.72[+$]	0.16±0.01
DM[7]	8.57±0.80[*+#~$^]	50.7±3.54[*+#~^]	25.2±7.68[*]

Data are mean±SEM.*significantly different to [1], [+]significantly different to [2], [#]significantly different to[3], [~]significantly different to [4], [$]significantly different to[5], ^significantly different to [6], "significantly different to[7].

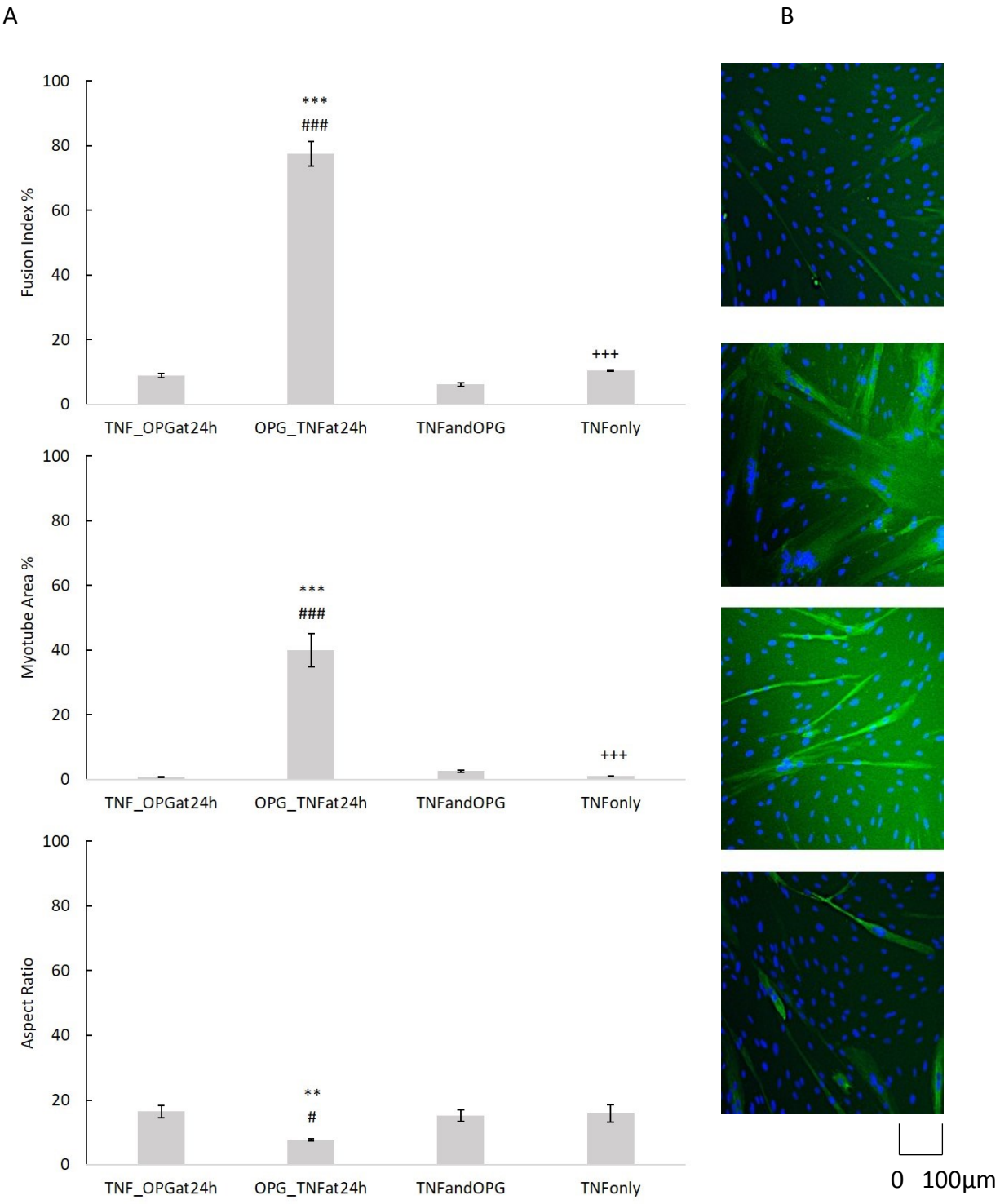

Figure 6.2; Differentiation parameters calculated at 60h of incubation. Panel A; Graphs Top to Bottom represent differentiation parameters; significantly different at ***p<0.0001 and **p<0.01 to TNFα_OPG AT 24h. Significantly different to OPG_TNFα at 24h at +++ p<0.0005, significantly different to TNFα and OPG at ###p<0.005, #p<0.05. Panel B represent; Top to bottom, immunohistochemistry staining for TNFα_OPG at 24h, OPG_TNFα at 24h, TNFα and OPG, TNFα only. Green shows differentiated myotubes, blue hows nuclei. TNFα was added at a concentration of 30ng.ml^{-1} and OPG was added at a concentration of 20ng.ml-1. Data are mean ± SE.

6.1.4.3. Objective 3

Human myoblast cells were cultured with the previously established (Objective 1) concentration of TNFα (30 ng.ml^{-1}) and with DM only. These cultures were ceased at 24,48,72 and 96 hours. The various concentrations of OPG found within the supernatant of these cultures are displayed in Figure 6.3. An effect of TNFα was found with univariate analysis F; (15,24) 4.931, p=0.003. There was also an interaction effect between TNFα and time F;(15,24) 3.340, p=0.004.

Simple main effects by pairwise comparison are displayed in Table 13. There was a significant difference at 24h for TNFα compared with DM only (p=0.001). At 48h a significant difference of OPG secreted was found for TNFα cultures compared with DM (p=0.003). At 72h, TNFα cultures differed significantly, albeit a lower concentration, from DM cultures (p=0.020), but at 96h no significant difference between TNFα and DM cultures was found.

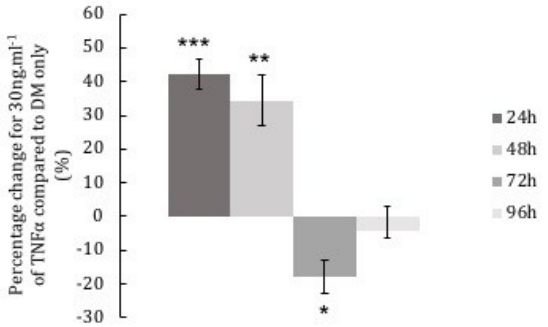

Figure 6.3; The percentage change of OPG concentration when cultured with a concentration of 30ng.ml^{-1} of TNFα compared to 0ng.ml^{-1} of TNFα. Baseline is zero when cultured with Differentiation media only. Error bars display standard error of the mean. Significantly different to baseline (*p<0.05, **;p<0.01,***p<.0001). Two repeats for each culture were carried out.

				95% Confidence Interval	
Time (Hours)	TNFα Concentration ($ng.ml^{-1}$)	OPG concentration ($pg.ml^{-1}$)	Significance p (Simple main effects compared to $Ong.ml^{-1}$ of TNFα)	Lower Bound	Upper Bound
24	0 30	617±17.7 877±10.2	**0.001**	521 781	712 972
48	0 30	641±6.36 861±19.1	**0.003**	545 765	736 956
72	0 30	918±1.84 755±2.73	**0.020**	822 659	1014 851
96	0 30	734±7.05 703±14.6	0.639	638 607	829 798

Table 14; Simple main effects displayed for differences in concentration of OPG secreted when cultured with different concentrations of TNFα at different time points. Data are mean ±SEM.

As well as investigating the concentration of OPG released by myoblasts when incubated with TNFα at $30ng.ml^{-1}$, a multiplex was used to identify selected myokines that may also be released in response to the culturing with TNFα, compared with no TNFα (DM only). It was found that osteonectin, LIF, IL-6 and FAPB3 showed significant changes. Interaction effects were seen for all four myokines; Osteonectin; F (15,24) 7.45, p<.0005. LIF; F (15,24) 15.7, p<.0005. FABP3; (15,24) 14.5, p<.0005. IL-6 F (15,24) 14.5, p<.0005. Pairwise comparison was used to identify simple main effects, between the concentration of TNFα and the concentrations of the myokines released. The results are found in table 3 with significance shown at p<0.05. Osteonectin showed significant difference at 72h and 96h with TNFα concentration of $30ng.ml^{-1}$ (p<0.020). LIF showed significant differences at all time points (p<0.004). FABP3 only showed a significant difference at 96h (p<0.0005). Finally, IL-6 showed significant differences at 48 and 96h (p<0.0005).

						Significance (Simple main effects compared to 0ng.ml^{-1} of TNFα)			
Time (Hours)	TNFα (ng.ml^{-1})	Osteonectin (ng.ml^{-1})	LIF (pg.ml^{-1})	FABP3 (pg.ml^{-1})	IL-6 (pg.ml^{-1})	Osteonectin P value	LIF P value	FABP3 P value	IL-6 P value
24	0	427±57.5	0±0	169±14.6	0±0				
	30	196±8.90	38±1.98	123±5.03	150±12.9	0.066	**0.004**	0.960	0.733
48	0	203±0.08	0±0	944±48.8	3±0				
	30	233±30.1	98±6.70	854±101	4082±985	0.807	**<.0005**	0.923	**<.0005**
72	0	530±16.1	0±0	2308±236	0±0				
	30	132±2.63	41±4.41	5153±555	821±5.43	**0.020**	**0.002**	0.142	0.071
96	0	793±201	18±5.48	11720±1155	6±0				
	30	127±0.14	70±26.9	6031±327	1868±293	**<.0005**	**<.0005**	**<.0005**	**<.0005**

Table 15; Simple main effects displayed for differences in concentration of myokines secreted when cultured with different concentrations of TNFα at different time points. Data are mean ±SEM

6.1.5 Discussion

The primary aim of this original research study was to identify if OPG protects against the effects of TNFα on human myoblasts in vitro. This was done through three main objectives stated in section 6.1.2.

Optimisation experiments did not identify any differences between concentration of TNFα or OPG and the myotube area percentage. Previous literature was used as a guideline for concentrations to be used in further experiments [17, 305].

Differentiation parameters, fusion index (%) and myotube area (%) were significantly greater when human myoblasts were subject to OPG adding TNFα after 24 hours (Figure 6.2a), compared with myoblasts cultured with TNFα adding OPG at 24h, TNFα alone and when adding TNFα and OPG at the same time.

As shown in the images in Figure 6.2b, it is clear the see the large variation in differentiation in these different culture models. If TNFα is added to the media on its own there are very few myotubes differentiated (less green illuminated in immunohistochemistry, figure 6.2b), whereas when adding OPG first and then adding TNFα after 24h there is clearly a significantly greater amount of differentiation, as shown from our statistical analysis, figure 6.2a. From this data it is indicated that OPG may be providing an initial 'protective' mechanism if added to the media before TNFα. OPG has previously been shown to have protective mechanisms using C2C12 cells of mice with muscular dystrophy, an OPG immunoglobulin fragment complex can improve muscular force, up to 54%, and reduce levels of inflammation [279]. Previous literature has focused on C2C12 cells or mouse models, this experiment shows the benefits of OPG can also be applied to human muscle cells *in vitro*.

To investigate the mechanisms of OPG in response to TNFα further co-culture models were used. It is known that OPG has been shown to be released from muscle cell cultures using C2C12 cells in response to immunoglobulin fragment complex [279], as stated previously. Here it is shown OPG is be released from human myoblast cells in culture in response to TNFα (Table 14). There has been speculation as to how it is that these muscle cells respond to TNFα in this protective manner and it has been assumed that the RANK/RANKL/OPG pathway is the key regulator. However, recent findings have shown this may not be the case [306]. Dystrophin/RANK double deficient mice, i.e. models that cannot stimulate through the RANK/RANK, pathway, are still able to respond to an OPG fragment complex and induce increases in muscular force [306]. This does not deny the fact that OPG is still able to protect against TNFα, a marker of inflammation that is associated with sarcopenia and bone ageing [134, 144, 146, 147]. These culture models would indicate that OPG, a well-known circulating bone marker [272, 294-296] is able to be released from human muscle in response to a marker of inflammation, which could therefore become a potential therapeutic target for ageing muscle. TNFα also has a large influence in cardiomyopathy and causes decline in cardiac muscle function [298, 301], OPG could act as a protective role in the same way as shown here, making it a clear option for intervention.

With the plates being cultured at different time points, it allows the pattern of OPG secretion to be seen, as shown in Figure 6.3. At 24h there is a significant increase compared to culturing with DM only. At 72h, a significant decrease, with 96h showing no significant change. This suggests that the majority of OPG is released during the first 24 to 48h in response to TNFα, and then OPG secretion begins to decline due to,

perhaps, another factor, although this cannot be certain. Further investigation is needed to identify the specifically activated signaling pathways, at these different time points.

Further analysis has shown other myokines are released from muscle in response to TNFα. Table 15 displays the findings of our multiplex analysis, highlighting four myokines to be measured in the supernatants of the culture models, described above. Osteonectin, Leukemia inhibitory factor (LIF), Fatty acid binding protein (FABP) and IL-6 were shown to be released from the muscle at different time points.

Osteonectin had the most significant differences at 96 h. Given that the majority of the changes in OPG happened during the first 24-48h, this could suggest that the Osteonectin is being taken up in response to the build-up of OPG, as there is a decline compared to baseline values. Osteonectin is a bone matrix glycoprotein that is abundantly expressed in bone [307]. Osteonectin deficient mice have shown to be severely osteopenic, highlighting that osteonectin is a positive regulator of bone formation [307], acting in a similar fashion to OPG [250]. It may be that Ostenectin is the secondary responder to OPG, taken up by the muscle in response to TNFα. The majority of FABP was also taken up at 96h, as there is a decline in concentration compared to baseline values. FABP's comprise of a large number of 15kDa proteins and they facilitate the transport of long chain fatty acids across the plasma membrane [308]. Exercise training, combined with low glycemic diets have been shown to increase insulin sensitivity and fat utilization [309], allowing fat to be ultilised as fuel for the training. Here the FABP seems to be increased in response to the OPG production after 96h of incubation. This gives further evidence that FABP is another

key participant in bone-muscle cross talk, here responding once OPG secretion has significantly declined.

LIF has shown significant differences at all time points. The secretion could, therefore, be in response to either OPG or TNFα. LIF is essential for embryo implantation in the mouse [310]. Its receptor (LIFR) has also been implicated to be involved the regulation of bone formation [307], through binding with Oncostatin M. There is little evidence available to show that LIF can be released from muscle in response to inflammation markers or circulating bone factors. Our findings show that LIF can be released from human muscle cells and another factor influencing bone-muscle cross talk. Finally, IL-6, showed the most prominent secretion at 48h and 96h. This fits with previous findings that show IL-6 to be released from muscle in response to exercise and inflammation [179, 182, 311].

Further research should be directed to identify the signaling pathways that are activated within the muscle cells to secret OPG, in response to TNFα, along with LIF, Osteonectin, FABP and IL-6. As well as the signaling pathways, any interaction at the receptor level of TNFα molecules would help to identify what these circulating factors do to the myoblast cells to protect the muscle cells from apoptotic effects of TNFα its action.

7.1 Chapter 7: General Discussion

The overarching aim of the PhD project was to provide novel insights into how exercise and circulating bone regulatory factors may influence both bone and muscle health within older age. This was addressed through four objectives:

1. Investigate the relationship between BMD and lean mass in athletic older people, taking into account accelerometry data, the training age and type of athlete (sprint or endurance).
2. Identify the circulating bone remodelling factors associated with BMD in healthy young and older adults.
3. Identify the circulating bone remodelling factors associated with whole body lean mass in healthy young and older adults.
4. Using the selected circulating factors identified in objective 2 and 3, investigate the responses of cultured human immortalised myoblasts after exposure to these factors.

7.1.1 Overview

Chapters 3-6 cover the original research carried out as part of this PhD, each addressing one of the objectives stated above, fulfilling the overall aim.

Chapter 3, objective1, revolved around a sample of exceptionally athletic older people and non- active healthy old. Comparisons of bone health and muscle strength were made between those active and non-active, as well as within the active group, between sprinters and endurance. The main findings here showed that sprinters had a higher BMD at the hip and spine than those competing in endurance activity and those not competing at all. On some occasions those carrying out endurance activity have no

greater bone health than those not competing. The differences in BMD between groups could not be explained by muscle mass or function, further research is needed to investigate these changes. Further studies are required to identify to what extent discipline-specific advantages in BMD relate to pre-existing differences in skeletal health, or to variance in skeletal loading not captured in this study.

Chapter 4, objective 2, aimed to identify any associations between the circulating factors; DKK1, FGF23, OC, OPG, OPN and sclerostin with whole body bone mineral (WBMD) density. It was found that Sclerostin, DKK1, OPG and OC were each positively associated with WBMD in older adults, despite the average WBMD being lower and circulating DKK1, OPG and sclerostin being higher in old than young. Multiple linear regression identified BMI, circulating sclerostin and whole-body lean mass as explaining, approximately, 14% of all variation on WBMD amongst older adults. This helped to refine the number of circulating factors that could be associated with both bone and muscle during ageing.

Chapter 5, objective 3, was designed to investigate those circulating factors and how, this time, they may be associated with whole body Lean mass, as well as WBMD. The same statistical procedure was used to identify associations with WB Lean mass, as used in Chapter 4. It was identified that OPG accounted for 12% of the variation in WB Lean mass, in the old. Further to this, we were able to carry out additional analysis between OPG and TNFα. When using TNFα as a covariate, OPG was significantly inversely correlated with WB Lean mass in both young and old. Whilst this chapter only represents statistical analysis, it confirmed two circulating factors, TNFα and OPG, that can interact with one another and in doing so OPG may be able to interact between

muscle and bone and influence the age-related decline in muscle mass and bone strength. Thus, this leads into the final chapter where these two factors are investigated at the cellular level using human myoblast cells, Objective 4.

In order to carry out cell culture models it was necessary to, first, find optimum concentrations of OPG and TNFα that the cells could be seeded at, this was identified at 20ng.ml^{-1} for OPG and 30ng.ml^{-1} for TNFα. Once concentrations were confirmed, the human myoblast cells could be cultured with the two factors, adding OPG and TNFα together and at varying time points. As shown in Chapter 6, Figure 6.2, there were clear differences between models used, with OPG showing to provide a "protective" mechanism against TNFα, allowing the myoblasts to differentiate, when OPG is added to the cultures before TNFα. As it was found here that some interaction was existing between the TNFα and OPG at the muscular level, the next stage of experiments were carried out to identify if OPG was able to be secreted from the muscle cells in response to the TNFα. Using 30ng.ml^{-1} of TNFα it was shown that the myoblast cells were able to secret OPG, in particular during the first 24h of incubation. Along with OPG other myokines were released; Osteonectin, LIF, FABP and IL-6. This chapter identified several molecules that are able to interact with both bone and muscle and in particular identified OPG as a potential therapeutic target against inflammation in older age.

7.1.2 Summary of main findings

The research carried out as part of this PhD has achieved the four objectives stated in section 2.2, addressing the overall aim; to investigate how exercise and circulating bone regulatory factors influence both bone and muscle health in older age.

These findings confirm that sprinting can cause greater increases in bone mineral density compared to endurance training, in older age. Endurance training does not

seem to produce any benefits to bone mineral density of the hip and spine, compared with non-active controls. The gains in sprinters comparatively to endurance master athletes cannot be explained by impact counts, muscle power or body composition. Circulating factors play a clear role in the bone mineral density and muscle mass of older adults. These differences seen initially between sprinters and endurance athletes could possibly be explained by variations in circulating factors, brought about by the specific training regimes. TNFα has been shown to have particularly detrimental effects during ageing and is associated with sarcopenia and chronic inflammation. It has been shown here that osteoprotegrin (OPG) can counter act these damaging effects of TNFα. It may be, within sprint training, OPG levels are somewhat elevated allowing OPG to act as a decoy receptor to the TNFα ligand. TNFα, therefore, is unable to stimulate its inflammatory and apoptotic pathways, reducing its negative effects, resulting in a slowed rate of the loss of muscle mass and bone strength as age progresses.

These findings add to current literature investigating exercise effects on bone in older age, identifying sprint training to have positive effects for the spine and hip, and that endurance running may not be the best exercise to slow the age-related loss of bone mass. Osteoprotegrein has been discovered to be a key factor that can influence both bone and muscle in older adults and can act in a protective mechanism from another factor TNFα. Osteoprotegrin could be targeted for further therapeutic intervention to slow down the loss of bone and muscle mass during ageing, and reduce chronic inflammation normally associated with ageing.

7.1.3 Further studies and limitations

Accelerometry data shown in Chapter 3 was not collected in the control population, if it were then a more comparable data set may have been better able to explain the differences in BMD between groups. The cross-sectional nature of the study limits assessment of causal relationships between type of sport and BMD due to possible uncontrolled confounders. There was very little information about other factors potentially related to bone health, such as use of medications and nutrient intake including vitamin D. In addition, a detailed training log was not taken, so there may have been some additional information missed, highlighting differences in exposure to higher impacts between sprinters and endurance runners. Master athletes may not be representative of the general ageing population, they have been training for the majority of their lives and they have avoided the age associated diseases. It cannot be certain that their physical activity is the reason they have avoided these diseases/comorbidities or if they are genetically predisposed. The findings in this chapter can only show the differences in BMD between sprinters and endurance master athletes. Longitudinal data collection is needed to be able to identify if sprinting in the general ageing population has the same effects on BMD, compared to endurance running, as shown in the master athletes

The circulating levels of markers, may be influenced by release from non-bone cells, so it is not possible to determine the originating cell type. It is possible that altered renal function can affect the levels of the circulating factors, but markers of renal function were not included in the present study due to limitation of plasma sample quantity. A phantom was not used to calibrate the DXA scanners across sites and we did not adjust the results to derive “standardised” DXA values, as others have done for hip and femur

sites [264]. Instead, all study centres followed the local quality control procedures, including use of phantoms and daily calibration and the results were adjusted for country of testing to account for possible systematic differences.

Within our cell culture models whilst we identified various factors that were released from the muscle it is not certain how they interact with the TNFα, OPG or at the cellular level of the muscle. Nor were we able to identify what was happening on the myoblast cells when OPG was added to the co cultures before TNFα to then allow the myoblasts cells to differentiate and be protected from the normal apoptotic effects of TNFα. Future studies should look to identify the specific interactions and pathways that occur at the muscular level. OPG has been identified as a potential therapeutic target for intervention with muscle ageing, however, without translation into an animal model, it cannot be certain that the interactions we have seen in culture will also be those that are seen within animal models. Using knock out OPG mice the levels of TNFα compared to controls would be able to show whether the interactions we have seen between OPG and TNFα are true.

7.1.4 Conclusion

This original research has shown circulating factors to have an influence on both bone and muscle, in particular OPG and TNFα. Muscle is able to secret OPG in response to TNFα as well as Osteonectin, LIF, FABP and IL-6, that are released as a response to TNFα and/or OPG. During ageing these factors could influence both bone and muscle deterioration. If these factors are further investigated they could become therapeutic targets of intervention to reduce the loss of bone strength and muscle mass during ageing. The most appropriate exercise regime suggested for ageing has not been well

defined, however, it has been shown, within this thesis, that sprinting, in master athlete, has more beneficial effects to bone health than endurance exercise and this should be taken into account when prescribing exercises for the elderly to slow down the effects of ageing on muscle and bone. The research conducted will contribute to the understanding of muscle and bone loss throughout older age and how these two organs may interact through circulating factors.

7.3. Appendix

Author	Year	Journal	Intro	Measurements	Intervention	Conclusion
Cunha et al	2018	Journal of Sport Science	Effects of Resistance Training with 1 or 3 sets of exercise on osteosarcopenia obesity older women	62 older women, aged 68, 12 weeks RT program	2 training groups performed 1 or 3 sets of resistance exercises 3 times weekly and control group. Measured muscle mass and bone with DXA.	No difference between training groups but Training groups showed a greater increase in muscle mass than controls. No effect on BMD between three groups. Overall higher positive change of Z scores in 3 set training group.
Beavers et al	2017	J Gerontol A Bio Sci Med Sci	To examine the effect of exercise during weight loss on hip and spine BMD, in overweight and obese older adults	Two, 5 month Randomised controlled trials, Calorie restriction with either aerobic or resistance training. 123 adults ages 69yrs- 67% female. Resistance training n=60 and aerobic N=63.	Resistance program involved 3 days per week of 8 body exercises, 3 sets of 10 reps at 70% 1RM, Aerobic training involved treadmill walking for 30 mins at 65-70% max hr. Measured BMD with DXA at baseline and 5 months	Total hip and femoral neck BMD was unchanged in the resistance training group and decreased in the aerobic training group.
Duckham et al	2015	Age and Ageing	To evaluate the effects of home (Otago Exercise Programme, OEP) and group (Falls exercise management, FEM) falls prevention exercise programs relative to usual care in older people	N=319 men and women aged 72yrs. Randomised into OEP, FEM or usual care, BMD and BMC and structural properties measured before and after 24 weeks of intervention	OEP group completed 58 mins per week of home exercise while the FEM completed 39 and 30 min per week of group and home exercise. For 24 weeks	No difference between groups in femoral neck BMD, or BMC at other skeletal sites. Exercise may need to exert higher strains for longer.

Elsisi et al	2015	Clinical Intervention Aging	Compare the response of BMC and BMD in elderly women to low frequency low plus magnetic field or circuit weight training (CWT)	12 weeks of intervention. N=30 aged 60-70 yrs. Randomly assigned to magnetic field or CWT.	12 Weeks of intervention, 3 times per week. Measured BMD and BMC of lumbar spine, femoral neck and trochanter	Both interventions showed statistically significant increases but Magnetic field showed slightly greater increase
Rantalainen et al	2011	Eur J Applied Physiology	To identify the effects of bi lateral hopping on bone markers	12 week bilateral hopping on balls of the feet. 25 men aged 72, assigned to control group or intervention group.	Subjects were tested in the lab three times per week for 12 weeks, conducting the bilateral hopping 5-7 times in sets of 10 seconds. Sets were performed at 7—90% intensity.	Bone markers, CTx Cicp bALP did not change from base line to 12 weeks in either groups
Marques et al	2011	Calcified Tissue International	Examine the effects of exercise against bone induced weakness	8 month multicomponent training with weight bearing exercises on BMD. N= 30 in exercise group and 30 in control group.	Training was performed for 60 mins 2x per week. Involved 10 min of warm up activities, 15 min of weight bearing exercises, 10 min muscular endurance, 10 min of balance, 10 min agility and 5 min of stretching. Carried out for 8 months	Femoral neck had an increase of 2.8% BMD
Sakai et al	2010	J Bone Mineral Metabolism	To test the effect of unipedal standing exercise on BMD of the hip in post menopausal women	N=94 Mean age 68 yrs, randomly assigned to exercise or control group. BMD of hip measured with DXA.	Exercise group performed the exercise three times a day for 6 months, with eyes open the participant was required to stand on one leg for one minute then the other leg= one set. One set was performed in the morning, noon and afternoon.	Percentages changes in the hip BMD between groups did not differ. Effects of exercise on BMD did show an effect only on participants over age 70 yrs .

Bocalini et al	2009	J Aging Health	Evaluate the effects of strength training on BMD of post menopausal women without Hormone replacement therapy	Subjects were randomized into training or no training groups.	24 weeks of strength training sessions three times per week . Measured BMD of lumbar spine and femoral neck	Demineralisation percentage was higher in the un trained group than trainer group, training attenuated the loss of BMD in post menopausal women.
Tolomio et al	2008	J Phys Act Health	Evaluate the effects on bone tissue and muscular strength of short term exercise program in post menopausal women	N=49 osteopenic/ osteoporotic women. Bone assessed by phalangeal quantitative osteosonography	Two groups exercise and control group. Supervised 20-week physical activity program that included aerobic, balance and strength training.	After training period, all bone parameters significantly improved in exercise group ($p<0.05$). No sig changes in control group.
Woo et al	2007	Age Ageing	Investigate the beneficial role of exercise in improving bone mineral density in elderly community dwelling participants	N=180 Subjects 90 men 90 women aged 65-74 yrs old	12 months. Subjects assigned to participate in Thai Chi or resistance training 3 times a week or no training. BMD was measured at baseline 6 months and 12 months	In women both the Thai Chi and resistance training had moderate lower loss of hip BMD compared to controls. No effects found in men.

Table 16; A systematic review of bone and exercise in the old. The literature was systematically reviewed using the search engine PubMed. The words and descriptors used for the data search were (Ageing or aging) AND (Bone) AND (Sport or exercise or Physical activity). The search was refined to include only publications within the last 20 years, the studies being randomised controlled trials (RCT) or Observational studies, participants aged 65 years or over and human studies only. The search returned a total of 72 papers. Out of the 72 papers returned, a total of 10 studies were appropriate for the systematic review because they described direct effects of exercise on bone.

Author	Year	Journal	Intro	Measurements	Intervention	Conclusion
Fielding et al	2017	PLoS One	To understand the effect of Physical activity on changes in physical function and the onset of major mobility disability. Lifestyle Interventions and Independence for Elders Study (LIFE)	Multi centre ranomised control trial followed for 2.6 yrs average. 1635 sedentary men and women aged 70-89 yrs.	Randomised to a structured moderate intensity walking, resistance and flexibility physical activity programme. Physical activity assessed by 7 day accelerometry and self report at baseline and 24 months. Outcomes included 400m gait speed, short physical performance battery test and onset of disability.	Small changes in physical activity improved 400 m gait speed and SPPB scores, which are used as indicators of frailty
Ossowski et al	2016	Clinc Interv Aging	Evaluate the impact of Nordic walking training on skeletal muscle index, muscle strength, functional mobility and functional performance in women with low bone mass	Participants were women aged 63-79 years with osteoporosis or osteopenia. Measurements taken with bioimpedance body analyser and muscle dynamometer.	Randomly assigned to control group or activity group. The activity group carried out the nordiac walking 3 times a week for 60 mins each session for 12 weeks. Measured at baseline at 12 weeks	Skeletal muscle mass, index and strength index of the knee extensor and flexor, all showed significant improvements. No change in the hang grip muscle strength or in the control group.
Landi et al	2016	BMJ Open	Evaluate the effect of sarcopenia on all cause mortality and the interaction among muscle loss, physical function impairment and multimorbidity on mortality risk over 10 years in community dwellers	All people within Sirente, Italy area n=364 aged 80 yrs+. Measured all cause mortality over 10 yrs, impact of physical function impairment asses using Short physical performance batter and multi morbidity on 10 year mortality risk	Sarcopenia was identified in 103 participants. 253 deaths recorded in 10years, 10 sarcopenic, 162 non sarcopenic. Of those, low physical performance levels had a greater mortality rates.	Physical function impairment is predictive of mortality in this region. In sarcopenic older persons, interventions to reduce functional decline may reduce mortality rates.
Mijnarends DM	2016	Age and Ageing	Physical activity may slow the rate of muscle loss and the incidence	People within Reykavik Area n=2309 Aged 66-93yrs old. Sarcopenia was	n/a	Sarcopenia at baseline was 7.3% and 16.8 % at follow up. Those reporting higher

			of sarcopenia. The aim was to examine the association of Physical activity with sarcopenia over 5 years.	identified using muscle mass, grip strength and gait speed. Amount of activity was assessed by self reported questionnaire.		levels of physical actvity had significantly lower likelihood of sarcopenia.
Nunes et al	2016	Age (Dordr)	Evaluated the effect of resistance training volume on muscular strength and on indicators of abdominal adiposity in post menopausal women	Muscle strength was measured and indicators of abdominal adiposity. No difference at baseline between groups	N=32 average age 61yrs. 11 in control group doing no exercise, 10 in low volume resistance training group and 11 in high volume resistance training group. 8 exercises were performed at 70% of 1 rm 3 times a week. For 16 weeks	In both trained groups showed increase in muscular strength and fat % reduction after intervention. Some differences between trained groups in indictors of adiposity
Zdzieblik et al	2015	Br J Nutr	Protein supplementation in combination with resistance training may increase muscle mass and strength in elderly subjects	53 male subjects aged 72.2 yrs. With sarcopenia completed as a randomized controlled study. Measured FFM FM and bone mass before and after intervention with DXA. Isometric quad strength was determined with standardized one leg stabilization test	12 week guided resistance training programme with here sessions a week. Supplemented either with collagen or a placebo.	Fat free mass and bone mass showed significant changes, Higher isometric quad strength all in group taking collagen supplementation
Lauseen et al	2015	J Am Geriatric Society	Progressive resistance exercise is used in patients to improve muscular strength and physical activity in older adults, but it is unknown if this will improve physical function.	Measure changes in physical function before and after 6 month progressive resistance exercise	Community dwelling older adults aged 70-85yrs, 6 month programme, Physical activity was measured with an activity monitor, physical function was assessed using short physical performance battery and repeated chair rise, stair climb and the 400m walk test. The	There were significant improvements in all measures of physical function from baseline to six months, no change in physical activity from baseline to 6 month . Other factors need to be targeted to improve physical activity

					resistance training was done 3 times a week, intensity progressed from two sets of 10 reps at 80% of 1RM to three sets of 12. Measured at baseline to 6 months.	
Reid et al	2015	J Gerontol A Biol Sci Med Sci	We compared two different lower extremity power training interventions on changes in muscle power, physical performance, neuromuscular activation and muscle CSA in mobility limited older adults.	52 subjects aged 78yrs.	16 weeks of progressively high velocity resistance training or high external resistance training. Two times a week for both groups Completing leg and knee extension exercises at max velocity.	Improvements in neuromuscular activation, short physical performance battery and leg extensor. There were no significant between group differences.
Shahar et al	2013	Clinc Interv Aging	To determine the effectiveness of exercise intervention and protein supplementation alone or in combination	65 sarcopenic elderly participants aged 60-74yrs. Assigned to control group, exercise group, protein supplementation or a combination.	60 mins of exercises twice a week for 12 weeks	The exercise programme was to found muscle strength and body composition, while protein supplementation reduced body weight and increased upper body strength among sarcopenic elderly.
Hanson et al	2009	J Strength and Conditioning	To investigate the effects of strength training on physical function and the influence of strength, power, muscle volume and body composition on physical function.	Healthy inactive adults aged 65 years and older. Underwent strength, power, total body composition and physical function testing before and after 22 weeks of strength training.	10 week unilateral strength program using untrained leg as an internal control preceded 12 weeks of whole body strength training.	Strength, power and fat free mass increased significantly with strength training in overall group. Women improved in walking whereas men improved in stair climb. Strength training improves functional tasks

Harber et al	2009	Am J Physiol Regul Integr Comp Physiol	To assess the influence of aerobic training on muscle size and function.	12 week cycle ergometer training. Muscle volume measured by MRI. Muscle biopsies taken to determine size and contractile properties of slow and fast twitch fibres.	12 week cycle ergometer training, 20-45 mins of 3 or 4 sessions per week	Aerobic training inc type 1 fibre size. And type 1 peak power was elevated, while type 1 unaltered in size and power. Indicate that aerobic training improves muscle function through remodeling contractile properties at myofiber level.
Raue et al	2009	J Appl Physiol	To assess single muscle fibre contractile function and whole muscle characteristics before and after 12 weeks oh high intensity resistance training. In very old women, aged 85+	Young women acted as a control group. Whole muscle size using CT and strength by 1 rep max were asses before and after training.	12 weeks progressive resistance training programme, assessed size, peak force, velocity and power on VL MHC 1 and 11 type fibres. Training sessions were 2-3 times per week.	1 rep max increased in old and young, no increase in thigh muscle csa in old. Type two fibres improved in size, peak force and power but no change in type 1 fibres. No changes in MHC type 1 or type 2 in the older women, shows the old women have a blunted response to resistance training at muscle and cellular level
Reid et al	2008	Aging Clin Exp Res	Investigate whether high velocity high power training improved lower extremity muscle power and quality in functionally limited elders greater than slow velocity resistance training	N=57 Adults aged 74yrs Randomised into power, strength or control groups. Outcome measures include 1RM strength and peak power. Total leg lean mass was used to determine specific strength and specific PP.	Training performed 3 times per week for 12 weeks, subjects performed three sets of double leg press knee extension exercises at 70% of 1rm	Peak power and specific peak power increased similarly from baseline in power and strength compared to controls. Gains in leg press peak power were greater in power than other groups. Total leg lean mass didn't change in any group

Table 17; A systematic review of muscle and exercise in the old. The following section of the review has been conducted as a systematic review, using the search engine PubMed. The words and descriptors used for the data search were (Ageing or aging) AND (Sarcopenia) AND (Sport or exercise or Physical activity). The search was refined to inclusion with publication within the last 20 years, the studies being randomised controlled trials or Observational studies and including participants aged 65+. This search produced 705 papers. Due to the large number for a small section within a review the search was further refined to include those with free full text. This search returned 254 papers, 20 of which were suitable for inclusion in the systematic review.

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