Exercise habits and ageing health : exploring the associations of FGF21 and lung microbiome in glycaemic regulation, bone health, arterial stiffness and respiratory health

Lee, Shuen Yee

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**EXERCISE HABITS AND AGEING HEALTH: EXPLORING
THE ASSOCIATIONS OF FGF21 AND LUNG MICROBIOME
IN GLYCAEMIC REGULATION, BONE HEALTH,
ARTERIAL STIFFNESS AND RESPIRATORY HEALTH**

LEE SHUEN YEE

LEE KONG CHIAN SCHOOL OF MEDICINE

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LEE SHUEN YEE

Lee Kong Chian School of Medicine

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for the
degree of Doctor of Philosophy

2020

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

31/07/2019

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Date

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A/Prof Lim Chin Leong Fabian

Authorship Attribution Statement

This thesis contains material from papers published in the following peer-reviewed journals in which I am listed as an author.

Chapter 3 is published as Lee SY, Burns SF, Ng K, Stensel DJ, Zhong L, Tan F, Chia KL, Fam KD, Yap M, Yeo KP, Yap EPH, Lim CL. FGF21 mediates the associations between exercise, ageing and glucose regulation. *Medicine & Science in Sports & Exercise*. 2020 Feb;52(2):370-380. doi: 10.1249/MSS.0000000000002150.

The contributions of the co-authors are as follows:

- I wrote the manuscript and performed the laboratory work, planned and executed the trials and collected the data at Lee Kong Chian School of Medicine. I analysed and interpreted the data.
- A/Prof Stephen Burns and Dr Frankie Tan provided inputs on exercise and health research and supervised some of the experimental trials. A/Prof Stephen Burns also provided inputs on the manuscript drafts.
- Dr Kenneth Ng provided inputs on medical clearance protocols and supervision of some of the experimental trials.
- Prof David Stensel provided guidance on exercise and metabolic health and edited the manuscript drafts.
- Dr Zhong Liang provided inputs on cardiovascular health research.
- Chia Kar Ling, Fam Kai Deng and Margaret Yap assisted in the planning of the trials and data collection.
- Dr Yeo Kwee Poo provided inputs on statistical methodology and analysis.
- A/Prof Eric Yap assisted in providing the initial project direction.
- A/Prof Lim Chin Leong provided the initial project direction, supervised the planning and execution of the experimental trials and data analysis and edited the manuscript drafts.

Chapter 4 is in preparation for manuscript submission as Lee SY, Burns SF, Ng K, Stensel DJ, Zhong L, Tan F, Chia KL, Fam KD, Yap M, Yap EPH, Lim CL. Pulse wave

velocity is associated with oxidative stress in ageing but not FGF21–adiponectin axis regardless of exercise status. Scientific Reports.

The contributions of the co-authors are as follows:

- I wrote the manuscript and performed the laboratory work, planned and executed the trials and collected the data at Lee Kong Chian School of Medicine. I analysed and interpreted the data.
- A/Prof Stephen Burns and Dr Frankie Tan provided inputs on exercise and health research and supervised some of the experimental trials.
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- Dr Zhong Liang provided inputs on cardiovascular health research.
- Chia Kar Ling, Fam Kai Deng and Margaret Yap assisted in the planning of the trials and data collection.
- A/Prof Eric Yap assisted in providing the initial project direction.
- A/Prof Lim Chin Leong provided the initial project direction, supervised the planning and execution of the experimental trials and data analysis and edited the manuscript drafts.

Chapter 5 is in preparation for manuscript submission as Lee SY, Chia KL, Fam KD, Yap M, Chotirmall SH, Yap EPH, Lim CL. Age-related bone loss is associated with FGF21 but not IGFBP1 in healthy adults. Experimental Physiology.

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- I wrote the manuscript and performed the laboratory work, planned and executed the trials and collected the data at Lee Kong Chian School of Medicine. I analysed and interpreted the data.
- Chia Kar Ling, Fam Kai Deng and Margaret Yap assisted in the planning of the trials and data collection.
- Asst Prof Sanjay Chotirmall provided inputs on study design and operation.
- A/Prof Eric Yap assisted in providing the initial project direction.

- A/Prof Lim Chin Leong provided the initial project direction, supervised the planning and execution of the experimental trials and data analysis and edited the manuscript drafts.

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- I wrote the manuscript, designed the study, operated the trials and prepared the samples for sequencing at Lee Kong Chian School of Medicine. I analysed and interpreted the data.
- Dr Micheál Mac Aogáin supervised and supported the analysis of the sequencing results and gave inputs to the direction of the manuscript. Dr Micheál Mac Aogáin and Nur A'tikah Binte Mohamed Ali performed the 16s rRNA sequencing.
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- A/Prof Eric Yap assisted in providing the initial project direction.
- Asst Prof Sanjay Chotirmall provided the initial project direction and edited the manuscript drafts.
- A/Prof Lim Chin Leong provided the initial project direction, supervised the planning and execution of the experimental trials and edited the manuscript drafts.

16 Jan 2020

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Figure 5.7 Summary schematic showing the significant associations between lung microbiome with lung function and arterial stiffness, within the young and elderly groups.

Abbreviations

AGE	Advanced glycation end-product
AIx	Augmentation index
AKT2	Serine/threonine protein kinase
ANOVA	Analysis of variance
AP	Augmentation pressure
ATF4	Activation of transcription factor 4
AUC	Area under the curve
BAP	Bone-specific alkaline phosphatase
BAT	Brown adipose tissue
BH4	Tetrahydrobiopterin
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
BP	Blood pressure
ChREBP	Carbohydrate-responsive element-binding protein
COPD	Chronic obstructive pulmonary disease
CTx	C-telopeptide cross-linked collagen type I
DEXA	Dual energy X-ray absorptiometry
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial NO synthase
FEV1	Forced expiratory volume in 1s
FFA	Free fatty acids
FGF21	Fibroblast growth factor 21
FGFR	Fibroblast growth factor receptor
FVC	Forced vital capacity
GLUT4	Glucose transporter type 4
HDL-C	High-density lipoprotein cholesterol
HMW	High molecular weight
HOMA-IR	Homeostatic model assessment of insulin resistance
IGF1	Insulin-like growth factor 1
IGFBP1	Insulin-like growth factor binding protein 1

IGT	impaired glucose tolerance
IL	Interleukin
ISI	Inverse Simpson index
KLB	β -klotho
LDL-C	Low-density lipoprotein cholesterol
LIRKO	Selective hepatic inactivation of insulin receptors
LRT	Lower respiratory tract
MET	Metabolic equivalent task
MI	Matsuda index
NADPH	Nicotinamide adenine dinucleotide phosphate oxidase
NGT	Normal glucose tolerance
NO	Nitric oxide
NTx	N-telopeptide cross-linked collagen type I
OA	Old active
OD	Optical density
OGTT	Oral glucose tolerance test
OPG	Osteoprotegerin
OS	Old sedentary
oxLDL	Oxidized low-density lipoprotein
PAGAC	Physical activity guidelines advisory committee
PCoA	Principal coordinate analysis
PP	Pulse pressure
PPAR	Peroxisome proliferator-activated receptor
PWV	Pulse wave velocity
RANKL	Receptor activator of nuclear factor kappa-B ligand
ROS	Reactive oxygen species
SD	standard deviation
SDI	Shannon diversity index
T2DM	Type 2 diabetes mellitus
TC	Total cholesterol
TG	Triglyceride
TNF- α	Tumour necrosis factor α
TRAP5b	Tartrate-resistant acid phosphatase 5b

URT	Upper respiratory tract
WAT	White adipose tissue
WC	Waist circumference
YA	Young active
YS	Young sedentary

Summary

Advancing age causes progressive decline in physiological functions and increases the risk of diseases and death. Age is an independent predictor of non-communicable chronic diseases such as bone fragility and respiratory and metabolic diseases, including cardiovascular disease and type 2 diabetes mellitus (T2DM). Besides age, there is good consensus among researchers and professional institutions that physical inactivity is another important factor that increases the risk of age-associated chronic diseases. Contralaterally, there is much research evidence supporting the effects of exercise in preventing and mitigating the effects of age-associated chronic conditions. Age-associated chronic disease accounted for ~71% of deaths globally and with the projected two-fold increase in elderly population worldwide by 2050, age-associated chronic disease-related mortality rate is also projected to increase exponentially in the next three decades. The increasing burden of age-associated chronic disease globally implies the sub-optimal effectiveness of existing healthcare models and therapeutics in treating and controlling these conditions at the population level. While it is not possible to reverse the growth of older population, more can be done through biomedical research to understand better the mechanisms underlying the favourable effects of exercise on ageing health, which may lead to potential therapeutic targets for preventing or decelerating the risk of age-associated chronic diseases. There is growing interest in the therapeutic role of fibroblast growth factor 21 (FGF21) and the microbiome in age-associated conditions. The primary aim of this thesis was to investigate the associations of FGF21 with the effects of age and habitual exercise on glucose regulation, bone mass, oxidative stress and arterial stiffness. The thesis also explored the effects of age on lung microbiome and the associations with arterial stiffness and lung function. The aims of the thesis were investigated through a review of relevant research evidence and four studies.

The review of the literature discussed research evidence related to age-associated decline in important physiological functions, including the loss in bone mass, decline in glucoregulatory function and increase in arterial stiffness. The effects of exercise on glucose regulation and arterial stiffness was also discussed in the review. The potential mechanisms driving the age- and exercise-associated effects on glucose regulation, arterial stiffness and bone loss were also examined. The review discussed the potential role of the microbiome, especially in the lungs, and its impact on ageing health. The effects of ageing and exercise on FGF21, and the glucoregulatory and anti-oxidative function of FGF21, and its impact on bone loss was also reviewed in this chapter. The downstream mechanistic pathways of FGF21, including adiponectin and insulin-like growth factor binding protein 1 (IGFBP1) were also discussed in the review. Although the evidence supports that FGF21 and the lung microbiome play important roles in metabolic, bone and respiratory health, none of the studies have investigated whether FGF21 and the lung microbiome were associated with age- and exercise-related changes in physiology, especially in healthy people.

The aim of study 1 was to investigate the association between FGF21 and glucose regulation, under the influence of exercise habits and ageing in healthy individuals. The hypothesis that glucose regulation with habitual exercise and ageing are associated with FGF21 is supported. In a cross-sectional study design, the concentrations of blood glucose and plasma FGF21, insulin and adiponectin during an oral glucose challenge were compared between 80 healthy individuals across young active (YA), young sedentary (YS), older active (OA) and older sedentary (OS) groups. FGF21 and insulin concentrations during the oral glucose challenge were lower in habitually active than sedentary individuals, independent of age. Ageing was associated with an increase in glucose and FGF21 concentrations during the OGTT, independent of exercise habits. Adiponectin concentrations were not affected by age or habitual

exercise. The results imply that FGF21 may modulate the associations between ageing and habitual exercise on glucose regulation, independent of adiponectin.

The aim of study 2 was to investigate the FGF21-associated pathways in arterial stiffness, under the influence of ageing and habitual exercise in healthy individuals, in the same cross-sectional groups and participants as Study 1. A secondary aim of this study was to investigate the associations of FGF21 with oxidative stress and arterial stiffness. The plasma concentrations of FGF21, adiponectin and oxidized low-density lipoprotein (an oxidative stress marker), and arterial stiffness measures were compared between 80 healthy individuals in YA, YS, OA and OS groups. Oxidative stress and arterial stiffness were higher in older than younger adults, despite no difference in FGF21 and adiponectin concentrations between younger and older groups, independent of exercise habits. Oxidative stress and arterial stiffness were also not different between habitually active and sedentary individuals, but FGF21 was associated positively with arterial stiffness in the sedentary group only, independent of age. In contrary to my hypotheses, these results imply that ageing, but not self-reported exercise habits, likely increases oxidative stress and arterial stiffness. The hypothesis that FGF21-associated pathways are related to oxidative stress and arterial stiffness is defeated.

Study 3 investigated the associations of FGF21 in bone turnover and decline in bone mineral density (BMD) during ageing, between 20 pairs of healthy younger and older family members. The hypothesis that FGF21 concentration is positively associated with age-associated bone loss is supported, but the involvement of IGFBP1 is not supported. Serum concentrations of FGF21, IGFBP1, receptor activator of nuclear factor kappa-B ligand (RANKL), bone formation marker bone-specific alkaline phosphatase (BAP) and bone resorption marker tartrate-resistant acid phosphatase 5b (TRAP5b) and BMD were measured in younger and older family pairs.

Compared with younger adults, older adults had higher FGF21 concentration, no change in IGFBP1 concentration, lower RANKL concentration, and higher concentrations of TRAP5b and BAP and lower BMD. These results imply that FGF21 is related to age-associated increase in bone remodelling and decline in BMD. Contrary to current evidence, IGFBP1 may not be a downstream effector of FGF21 and IGFBP1 is unlikely involved in RANKL-associated osteoclast differentiation in healthy humans.

Study 4 explored the age-associated changes in lung microbiome and their links with arterial stiffness and lung function in the same 24 pairs of healthy younger and older family members. Bacterial microbiome composition was determined from sputum samples and arterial stiffness and lung function measures were compared between younger and older family pairs. Ageing was associated with an increase in relative abundance of Firmicutes and a decrease relative abundance of Proteobacteria. Different microbes in the lungs were independently associated with lung function and arterial stiffness, in an age-dependent manner. The hypothesis that age-associated changes in lung microbiome composition is related with changes in the lung function and arterial stiffness during ageing is supported.

The results from these studies support that in healthy individuals, FGF21-associated pathways are related to age- and habitual exercise-associated effects on glucose regulation, arterial stiffness and bone mass. The data also suggest that the downstream pathway of FGF21 is not associated with adiponectin and IGFBP1. The exploratory study on the airway microbiome support that the lung microbiome is plausibly associated with age-related decline in physiological function. These results imply that FGF21 and the lung microbiome are important factors related to the normative age- and exercise-associated physiological changes in healthy individuals, which support their possibility as effective targets to prevent or decelerate age-

associated disease onset. Future studies should focus on elucidating the downstream mechanisms that drive the effects of FGF21 and lung microbiome on ageing health.

Chapter 1: Introduction and Literature review

1.1 Introduction

Ageing is characterised by the progressive loss of physiological integrity, leading to functional deterioration and increased vulnerability to disease and death (1). There are approximately 800 million people aged >60 years old globally, which accounts for ~11% of the population worldwide and this population is projected to more than double to >2 billion people (~22% of the population) by 2050 (2, 3). In Singapore, the proportion of elderly population, aged ≥ 65 years old, increased by 1.5-fold within 10 years, from 8.7% in 2008 to 13.7% in 2018 and is expected to make up 16% of the population by 2030 (4). The ageing population worldwide and in Singapore imposes a major healthcare burden as advancing age is an independent risk factor for chronic diseases, such cardiovascular diseases, type 2 diabetes mellitus (T2DM), chronic respiratory diseases and bone fragility (5-7).

Among the elderly population aged ≥ 65 years worldwide, cardiovascular diseases accounted for ~18 million deaths and respiratory diseases accounted for ~4 million deaths each year, while >130 million people suffer from T2DM currently (8-10). In musculoskeletal health, bone fragility affects about 200 million people globally, especially in the elderly population, which contributed to ~9 million fractures annually (11). These chronic diseases largely affect the elderly population and account for 41 million deaths each year, or 71% of deaths globally (12). Arterial stiffness contributes to the pathophysiology for a multitude of these age-associated chronic diseases e.g., heart failure, stroke, hypertension, retinopathy and coronary heart disease and T2DM (13, 14), and is associated with 15–45% increase in the risk of developing cardiovascular disease and cardiovascular and all-cause mortalities (15, 16). The acutely increasing ageing population globally and the associated increase in the prevalence of chronic

diseases impose significant burden not only on healthcare systems worldwide, but also on the labour force, social services and economy, which will continue to increase in the next 3 decades as the world ages (17). Although the increasing trend of the ageing population over the next three decades is a reality that cannot be reversed, more can be done to mitigate the effects of age-related chronic disease. Therefore, there is an urgent need to further understand the underlying factors driving the onset of age-associated chronic diseases that may lead to effective solutions to prevent or decelerate disease onset.

Physical inactivity is a known risk factor for the development of age-associated chronic diseases including T2DM, cardiovascular disease and bone fragility (18). Increased exercise reduces the likelihood of developing metabolic diseases, such as T2DM, and increases the likelihood of improving metabolic diseases in individuals at high risk of T2DM (19). A recent cross-sectional study of 96,706 participants found that compared with people with chronic disease, people without chronic disease had 9% or 61 min more moderate intensity activity and 11% or 3 min more vigorous intensity activity per week (20), which suggests the health benefits of being physically active. Physical activity and exercise have been touted as the “multi-potent pill” because the same dose of exercise is beneficial for a multitude of chronic diseases and protects against all-cause and cardiovascular disease mortality (21). The US Department of Health and Human Services commissions the Physical Activity Guidelines Advisory Committee (PAGAC) in 10-year intervals as an expert panel to conduct systematic review of scientific evidence and make recommendations on physical activity and health, to promote disease prevention and management (22). The second edition of the PAGAC report (2018) supported the recommendation for 75–150 min per week of moderate-to-vigorous intensity physical activity to effectively lower the risk of common chronic diseases and the risk of progression for a multitude of age-related chronic health conditions such as osteoarthritis,

hypertension and T2DM (22-24). The elderly should also participate in 2–3 strength training sessions weekly, comprising 2 sets of 8–12 repetitions of each exercise to protect and improve musculoskeletal health (25). In physically inactive adults, replacing sedentary behaviour with light intensity physical activity is likely to produce health benefits (26). The multi-system anti-ageing effects of exercise are also both therapeutic and preventive against bone fragility (27, 28) and improve tolerance to symptoms of respiratory diseases such as dyspnoea and fatigue (29-31). By attenuating the arterial stiffness that occurs during ageing, regular exercise also improves vascular health (32). Because exercise helps to prevent or manage age-associated chronic health conditions, whose prevalence is increasing progressively worldwide, there is an urgent need to understand better the mechanisms underlying the beneficial effects of exercise to provide the clue for potential therapeutic targets for preventing or decelerating the risk of age-associated chronic diseases.

The emerging role of the microbiome in mediating age-associated chronic diseases has been investigated primarily in the gut. The human body is host to trillions of microbes that colonize the body surface and which are collectively called the “core microbiome”. Loss of diversity in the core microbiota groups modulates age-related changes in immunity, frailty and metabolic activities, which may contribute to skeletal and metabolic disorders (33-35). The primary focus of research on the human microbiome during ageing is on the gut and its influence on host metabolism. Different systems such as the innate immune system recognise and monitor the presence of microbes in the body, affecting the composition of the microbiota. In healthy individuals, the greater gut microbiome diversity is associated with improved metabolism, while dysbiosis of the gut microbiome composition triggers inflammatory responses associated with diseased states (36). However, recent studies have also shown the presence of microbes in the respiratory tract that interacts with age-associated respiratory diseases (37). For example,

in patients with airflow limitation, a shift in lung microbiome towards Proteobacteria (mainly *Haemophilus*) is observed (38). The composition of the lung microbiome also differs in respiratory disease and in health (39). Compared with healthy people, people with restricted airflow and declining lung function have decreased diversity in the lung microbiome (39). Declining lung function is associated with arterial stiffness, which is a risk factor for cardiovascular mortality (40-44). Although the lung microbiome is linked to various age-associated chronic diseases (45), its associations with normative ageing is not known in healthy people. Understanding the relationship between lung microbiome and physiological changes during normative ageing would provide a reference point for comparison with diseased states.

Recent evidence suggests that fibroblast growth factor 21 (FGF21) could potentially play important mechanistic roles in the attenuation of age-associated chronic diseases. FGF21 is an endocrine hormone produced mainly by the liver in response to nutritional, physiological and pathological stimuli, and regulates oxidative stress and metabolism (46, 47). FGF21 also improves glucoregulatory and cardioprotective functions (48-50). Despite the favourable effects of FGF21, administration of FGF21 results in bone loss (51-53). FGF21 concentrations are also independently influenced by both ageing (54, 55) and exercise (56, 57), raising the possibility of FGF21 as a central mediator and therapeutic target of age-associated chronic diseases and the favourable effects of exercise. The literature review discusses these age-associated health conditions such as bone loss, declining glucoregulatory function and increasing arterial stiffness, and explores the associated role of exercise, the microbiome, particularly the lung microbiome, and FGF21 as a potential mediator of these age-associated chronic conditions.

1.1.1 Bone health

Musculoskeletal health is integral for mobility and function, which is essential in maintaining quality of life and improving cardiovascular function and musculoskeletal metabolism (58). A decline in musculoskeletal function is common with advancing age, which results in adverse health conditions, such as osteoarthritis and osteoporosis (59). Osteoarthritis is a cause of disability and is characterised by degeneration of joint cartilage, which leads to pain and loss of function. In older people aged 60 years and above, osteoarthritis affects 9.6% of men and 18% of women (60). Importantly, osteoporosis is a systemic skeletal disease that is associated with an increased risk of fracture and bone fragility and is one of the most common chronic disease in ageing populations, affecting ~10.3% of adults aged >50 years globally (61, 62). Given the huge musculoskeletal disease prevalence and burden, it is important to understand better the mechanisms that drive the age-related increase in bone fragility, in order for early intervention or developing therapeutics.

1.1.1.1 Definition of osteoporosis

Advancing age is an independent risk factor for osteoporosis because of the age-related loss of bone mass and declining bone mineral density (BMD), especially after the age of 50 years (63). Bone loss begin in women at the age 40–44, and in men at the age 25–39 (64). An average decline of BMD of 6.8% over 5 years was observed in females and a decline of BMD of 3.2% over 5 years was shown in males (64). BMD is a standard indicator of osteoporosis and its severity, and provides a quantitative measure of bone mass per unit area (g/cm^2). According to the World Health Organization, the diagnostic classification of osteoporosis is based on the T-score derived by dual energy X-ray absorptiometry (DEXA) scanning at the spine, hip or forearm. The main categories include normal BMD (T-score ≥ -1), osteopenia (T-score between -1 and -2.5), and osteoporosis (T-score ≤ -2.5) for men and postmenopausal women

aged ≥ 50 years (65). For premenopausal women and men aged < 50 years, a Z-score < -2 is considered to indicate a low BMD, and a Z-score > -2 indicates a normal BMD (65).

1.1.1.2 Ageing and bone remodelling

Gradual bone loss occurs during ageing despite the well-regulated bone remodelling process. Bone remodelling is a continuous physiological process initiated by signals from osteocytes embedded in mineralized bone matrix (66). “Old” bone mineral is removed during bone resorption by osteoclasts, which are specialized multi-nucleated cells derived from haematopoietic precursors (67). New bones are formed through mineralization of the bone matrix by osteoblasts, which are of mesenchymal stem cell lineage (67). The fine balance of osteoclast and osteoblast activity occurs through the repair of bone microdamage, regulation of bone mineral homeostasis and responses to mechanical loading (67, 68), and is essential for maintenance of bone turnover, skeletal regeneration and bone strength,

Given the importance of bone turnover and bone metabolism, biochemical markers of bone remodelling have been studied (69). These markers include bone-specific alkaline phosphatase (BAP) and osteocalcin for bone formation, and tartrate-resistant acid phosphatase 5b (TRAP5b) and N-telopeptide and C-telopeptide cross-linked collagen type I (NTx and CTx) for bone resorption (70). An increase in bone formation occurs during ageing, as evidenced by the steady increase in serum osteocalcin concentration every decade from age > 50 years in both men and women, regardless of race, although men have lower circulating serum osteocalcin concentration than women (71). Compared with women aged 20–49 years, postmenopausal women aged > 50 years exhibit a 62–67% higher serum osteocalcin concentration (71, 72). Serum BAP concentration is also 16–49% higher in postmenopausal than in premenopausal women, and increases from age 50 years and remains fairly constant with every decade increase

in age (71). In a 4-year longitudinal study of men aged ~48 years, serum BAP concentration increased by ~8% despite a decrease in bone mineral content and an increase in bone loss by ~2% (73). These results suggest that ageing is associated with an increase in bone formation markers such as BAP and osteocalcin concentration in both men and women, and that these increases reflect increased bone turnover.

An increase in serum CTx concentration, which is indicative of collagen degradation, predicted hip fracture risk in elderly women (aged ~81 years) during a 3-year follow-up (74). In another study, urinary CTx and NTx concentrations were higher in elderly women aged ~82 years than in premenopausal controls (75). In the same study, elderly women with hip fracture also had a higher CTx concentration than their age-matched controls without hip fracture, which suggests that bone resorption increases with age and is associated with bone fragility. Serum TRAP5b concentration, a marker of osteoclast formation, increased by 44% after total hip replacement in both men and women (aged ~63 years), which suggests that TRAP5b is indicative of the role of osteoclastic activity in bone remodelling (76). The increase in TRAP5b and CTx concentrations during ageing suggest that bone resorption increases because of the increase in osteoclastic activity. During healthy ageing, bone formation and resorption increase, but the bone resorbed by osteoclasts is not fully balanced by the bone formed by osteoblasts, and the end result of this imbalance is bone loss and fragility (77).

1.1.1.3 Mechanisms driving age-related bone loss

Oxidative stress increases with age and may influence bone turnover and lead to an age-associated decline in BMD and resultant bone loss (78-80). Reactive oxygen species (ROS) such as superoxide and hydroxyl radicals are generated as by-products of metabolism (78-80). During ageing, accumulation of ROS coupled with declines in activity of antioxidant enzymes,

such as superoxide dismutase and glutathione results in an increase in bone remodelling and lower BMD and bone strength (81, 82) (Figure 1.1). In mice, oxidative stress decreases osteoblast and osteoclast numbers, induces osteoblast apoptosis and proliferation, and suppresses osteoclast differentiation in bone (81). In the same study, anti-oxidant administration attenuated bone fragility and increased osteoblast survival. Mice with deletion of Forkhead box O transcription factors, which are involved in protection against oxidative stress, exhibited increased oxidative stress and bone loss caused by increased osteoblast apoptosis (83). These studies suggest that age-associated increases in oxidative stress could result in bone loss by affecting osteoblast and osteoclast function, as well as bone turnover.

Figure 1.1 Suggested mechanisms driving age-associated bone loss. Upward arrows indicate an increase and downward arrows indicate a decrease. Green boxes represent main mechanisms driving the age-associated increase in oxidative stress, while blue boxes represent bone turnover.

Age-associated increases in oxidative stress are linked to increased apoptosis of osteocytes, which sense bone microdamage and mechanical strain, and participate in bone repair and bone remodelling in mice (84). Compared with young mice, ageing mice exhibited lower BMD and bone strength at the spine and femur, and these decreases were associated with increased osteocyte apoptosis and oxidative stress (84). Receptor activator of nuclear factor kappa-B ligand (RANKL) is a key factor in osteoclast differentiation and function (85). RANKL binds to the RANK receptor on osteoclast lineage cells, and this binding activates osteoclasts and increases bone resorption (85). Increased osteocyte apoptosis with ageing results in increased production of RANKL by dying osteocytes, which initiates the remodelling process during bone microdamage (86). The inability of osteocytes to detect microdamage may affect bone resorption and formation, and may impair bone structural integrity, which could result in the increased bone brittleness and fracture risk observed during ageing (86).

An age-associated deficiency in sex steroids can also contribute to bone fragility and fractures in both women and men (87). Sex steroids control bone formation, resorption and turnover rate (87) (Figure 1.1). The decline in BMD with advancing age, especially during the early postmenopausal period in women, is largely attributable to the loss of oestrogen activity, which plays a key role in regulating bone metabolism (88). Oestrogen deficiency prolongs the lifespan of osteoclasts and shortens the lifespan of osteoblasts and osteocytes (89). During ageing, the loss of oestrogen increases bone resorption by osteoclasts, decreases bone formation by osteoblasts and activates bone remodelling (88). In men, androgen deficiency during ageing affects the maintenance of trabecular bone through the decline in activities of osteoblasts and osteocytes (90, 91). Compared with young mice, older mice had lower BMD and bone strength and increased oxidative stress, and these differences could be reversed by treatment with oestrogen and androgen both *in vitro* and *in vivo* (84, 92). These findings suggest that an age-

associated deficiency of sex steroids impairs the defence against oxidative stress in bone, which may lead to increased osteoblast and osteocyte apoptosis and increased bone resorption.

Lifestyle factors such as dietary habits can also influence the effects of ageing on BMD. Ageing causes deficiencies in the supply of calcium, phosphate and vitamin D, which impede bone formation and mineralisation, and increase bone resorption (93). Insufficient dietary intake of these nutrients can increase the magnitude of the decline in BMD with age. For example, a meta-analysis showed that increasing dietary calcium intake increased BMD by 0.6–1% at the total hip at one year, and increased BMD by 0.7–1.8% at the lumbar spine and femoral neck at two years (94). Inclusion of fruits, phytochemicals and anti-oxidants in the diet may attenuate the age-associated increase in oxidative stress and improve BMD, and higher intake of fruits and vegetables in humans is positively associated with increased BMD (95). Compared with the bottom tercile of fruit intake, participants belonging to the top tercile had an average of 3% increase in BMD score (95). In animals, consumption of fruits high in anti-oxidants increases bone mass, bone strength and trabecular bone volume through the amelioration of oxidative stress, increased osteoblast differentiation and inactivation of osteoclasts (96-98). These studies collectively suggest that consumption of calcium and food high in anti-oxidants attenuate oxidative stress, which is a central regulator of bone turnover and bone mass. In summary, the mechanisms driving the age-associated decline in BMD include non-modifiable endogenous factors such as increasing oxidative stress, osteocyte and osteoblast apoptosis, and sex steroid deficiency, as well as modifiable lifestyle factors such as diet (Figure 1.1).

1.1.1.4 Exercise and bone fragility

Weight bearing exercises are preventive and therapeutic against bone fragility especially in older adults, increasing bone mineral density (BMD) by ~1–2% (27, 28). In humans, a single

bout of moderate to maximal intensity endurance running exercise increased concentrations of markers of bone formation (BAP) and bone resorption (CTx) 3–4 days after exercise, suggesting that exercise can stimulate an increase in bone mineralization and turnover (99, 100). In another study, 3 sessions per week of strength training lasting 4 months in men (aged 54–61 years) increased lumbar spine BMD by 2% and femoral neck BMD by 3.8% (101). A 6-month exercise intervention that combined strength and endurance training in both men and women (aged 55–75 years) increased BMD, and this increase in BMD was positively associated with increased cardiorespiratory fitness, lean body mass and muscular strength (102). Physical activity induces mechanical load on the musculoskeletal system, which triggers an increase in osteocyte signalling to bone surface cells and activates bone remodelling (103, 104). During mechanical loading with exercise, osteocytes inhibit the secretion of sclerostin, which functions to stop bone formation, and this results in increased bone formation, bone strength and BMD (105, 106). Because of mechanical loading generated from exercise, an increase in osteogenesis and bone formation occur through the active canonical and non-canonical β -catenin signalling pathways (107). Exercise also reduces ROS production and attenuates oxidative stress, which results in a decrease in age-related bone loss through mechanisms such as decreased osteoclast-mediated bone resorption (107, 108). Collectively, these findings suggest that both acute and chronic weight-bearing exercise can improve and protect against the age-associated decline in BMD in older men and women, through the direct signalling to osteocytes for activation of bone remodelling, or oxidative stress-related pathways that result in increased bone formation and decreased bone resorption.

1.1.1.5 Genetic influences on BMD, bone loss and fractures

The genes that are inherited from the parents can determine about 50–85% of BMD and bone mass across all ages and skeletal sites (109, 110). In 724 postmenopausal female twins who

exhibited bone loss over a 5-year period, 40% of the variance in bone loss at the forearm and lumbar spine was genetically determined (111). Heritability of age-related bone loss in men has been studied less widely, but no heritable bone loss component at the wrist was found in 50 male twins over a 16-year follow-up (112). A family-based cohort reported a 25–44% heritable component to BMD at the spine, total hip and forearm in Mexican-American family members (all aged >45 years), even after adjusting for sex (113). These studies suggest that genetic influence play a major role in bone mass regardless of sex, and it is important to consider genetic influences on BMD when evaluating the mechanisms involved in age-associated bone loss.

The heritable component of bone mass is also evident in incidence of fractures. For example, the heritability of hip fracture was ~47% in Swedish twins younger than 79 years, and the heritability of wrist fracture was 54% in female twins and 25% in postmenopausal women and their first-degree relatives (114-116). Because bone mass and BMD are influenced by the balance between bone resorption and formation (61, 117), the similarity of BMD between family members may be reflected in the heritability of bone turnover markers (118). Markers for bone formation such as BAP and osteocalcin had a 74% heritability component in 1068 twin pairs who were predominantly females (119). Bone resorption markers such as CTx also had a 39–55% heritability component in postmenopausal twins aged 45–69 years (120). Although important, genetic factors do not fully account for bone mass, suggesting that other factors such as environmental factors are implicated. These environmental factors include diet and exercise habits that also reduce bone mass, by increasing oxidative stress (121, 122). Genetic and environmental factors are both important determinants of BMD and should be accounted for when investigating the mechanisms driving age-associated bone loss.

1.1.2 Glucose regulation

1.1.2.1 Ageing and glucose regulation

Ageing is associated with increased insulin resistance and glucose intolerance, and age is also an independent risk factor for glucose intolerance and T2DM (123). For example, one cohort study involving 3772 Caucasian people (aged 20–74 years) showed that plasma glucose concentration increased by 0.2–0.3 mmol/L per decade of life in both fasting samples and at 2-h time-point during an oral glucose tolerance test (OGTT) (5). The age-associated increase in insulin resistance is attributed mainly to impaired insulin secretion as a result of declining pancreatic β -cell function and loss of glucose-stimulated insulin secretion (124, 125). The age-associated decline in lean mass may also contribute to decreased peripheral glucose uptake into muscle, which is the major site of insulin-stimulated glucose uptake *in vivo* (124, 126, 127). The increase in visceral fat content with age also promotes secretion of inflammatory cytokines, including interleukins (IL)-1 ($\text{IL-1}\alpha$ and $\text{IL-1}\beta$) and IL-18, whose increased circulating concentrations are associated with T2DM and obesity in humans (128). Therefore, ageing is associated with decline in physiological function leading to impaired glucoregulatory function. The mechanisms linking advancing age to glucose dysregulation are multifactorial and complex, but is likely through changes in body composition, leading to insulin resistance and subsequent β -cell dysfunction. The pathogenesis for insulin resistance has been attributed to genetic factors and increasing chronological age, as well as physical inactivity that facilitate the progression of glucose dysregulation.

1.1.2.2 Exercise and glucose regulation

There is good consensus among researches that physical inactivity independently and in combination with ageing, contribute to the decline in glucoregulatory function (129, 130). Healthy non-exercising young men who decreased their daily step count by 87% per day (from

10,501 to 1,344 steps/day) for 2 weeks exhibited decreased peripheral insulin sensitivity (131). Every hour of sedentary behaviour was associated with a 22% increase in T2DM incidence among European people aged ~60 years (132). Conversely, each 500 kcal increase in weekly energy expenditure was associated with a 6% lower age-adjusted risk of developing T2DM (133). Exercise training appears to be equally beneficial for both people with normoglycaemia and hyperglycaemia. For example, 6 weeks of moderate intensity aerobic exercise training (4 × 20 min/week) improved whole-body glucose disposal by 38% in healthy participants and by 42% in insulin-resistant participants (134). In the 'diabetes prevention program', pre-diabetic and diabetic patients who participated in >150 min of weekly physical activity over 2.8 years had a 58% lower incidence of diabetes compared with the sedentary control group (135). Over a 10-year follow-up of the same participants, the incidence of diabetes in the exercise group was also 34% lower than in the control group, which suggested that the beneficial effects of exercise on glucose regulation persist for at least 10 years (135, 136). A joint position statement with the American College of Sports Medicine and American Diabetes Association concluded that an exercise frequency of ≥3 times per week, with a total duration per week of 75–150 min of moderate to vigorous intensity aerobic exercise is required to manage blood glucose and prevent diabetes (137). These findings indicate that exercise improves glucose regulation in both young and old people, and in those who are healthy or who have diabetes. Regular exercise habits therefore likely reduce the risk and incidence of T2DM and should be undertaken as part of lifestyle changes to prevent T2DM onset, especially in high risk adults.

Exercise improves glucose regulation by increasing glucose delivery, transport and metabolism, mainly in the skeletal muscles. Muscular contractions during exercise increase capillary recruitment and muscle blood flow by ~20-fold (138), which acutely increases oxygen and glucose delivery by 67% and skeletal muscle glucose uptake by ~60% (139). Exercise training

enhances the responsiveness of skeletal muscles to insulin by increasing expression of proteins involved in glucose metabolism and insulin signalling. For example, endurance exercise increases glucose transporter type 4 (GLUT4) gene and protein expression by ~30–52% (140) and translocation of GLUT4 to the plasma membrane by 2.8-fold (141), which increases glucose transport and uptake, and improves glycaemic control (142).

1.1.2.3 Interactive effects of ageing and exercise on glucose regulation

Increasing insulin resistance and visceral fat mass, as well as decreasing muscle mass are key factors related to the age-associated decline in glucose regulation. Exercise reduces the risk of T2DM in older adults by improving insulin resistance and body composition. Compared with sedentary people, exercise-trained people have better glucose tolerance (143, 144) and insulin sensitivity (143-146), as evidenced in cross-sectional studies using hyperinsulinaemic–euglycaemic clamps (145, 146) and OGTTs (143, 144).

The glucoregulatory response to habitual exercise training differs between younger and older groups (135, 143, 144). In one study, habitual exercise training decreased fasted blood glucose concentration by 7% in the older but not in the younger group (144). In young healthy adults, both chronic and acute bouts of exercise training did not alter glucose concentration and whole-body insulin sensitivity during an OGTT (147, 148). However, in elderly patients with T2DM, 6 months of combined aerobic and resistance training decreased fasting glucose concentration by 3% and 2-h glucose concentration by 24% during an OGTT, compared with pre-exercise concentrations (143). In the same study, 2-h insulin concentration during an OGTT was also 32% lower after exercise training (143). In non-diabetic older individuals (~50 years), increasing physical activity lowered the incidence of T2DM by 39% compared with pharmacological treatment (135). In people aged >45 years, 4–33 months of exercise

intervention increased muscle GLUT4 mRNA and protein expression by 30–52% and insulin sensitivity by 20%; these changes were reflected in a 58% reduction in the incidence of diabetes (135, 140). Taken together, the improvements in glucose regulation and insulin sensitivity with exercise seem to be more effective in older than younger adults. Physiological differences between young and older adults, including increased adiposity and decreased lean mass, and impaired β -cell function during ageing (124, 126, 128), may contribute to the differences in the effects of exercise on glucose regulation and insulin sensitivity. However, studies that directly compare the effects of habitual exercise on glucose regulation between younger and older adults, in the absence of chronic disease, are lacking.

1.1.2.4 Glucoregulatory mechanisms induced by exercise training in older adults

Ageing is associated with 46% decrease in β -cell insulin secretory capacity despite no change in β -cell glucose sensitivity (149), suggesting that insulin-dependent pathways are involved in mediating glucose regulation during ageing. The observed exercise-associated improvement in glucoregulatory function during ageing could be linked to insulin-dependent pathways (135, 140). A single exercise bout in old rats increased muscle sensitivity for insulin-stimulated glucose transport despite no effect on muscle GLUT4 protein content, suggesting that in animals, exercise improves glucose metabolism by improving muscle insulin sensitivity (150). In humans, insulin-dependent skeletal muscle GLUT4 protein content increased by ~1.7-fold after exercise interventions ranging from 1–12 weeks in older pre-diabetic and T2DM patients aged 44–67 years (151, 152). In older healthy people aged 40–65 years, 14 weeks of endurance exercise increased GLUT4 protein expression and content by 1.8-fold (153). However, muscle GLUT4 protein expression was not altered by either 12 weeks of ski training in older participants aged ~67 years (154) or 1 week of endurance exercise training in non-diabetic obese people aged ~48 years (151). These findings suggest that an increase in GLUT4

expression after exercise training is not consistently observed in older adults, which could be due to difference in health status and exercise of participants, or that other mechanisms apart from GLUT4 might be involved in exercise-related improvement in glucose regulation.

Exercise may also improve glucose regulation in older adults by increasing hepatic insulin degradation, which would reduce insulin resistance. In one study, an acute exercise bout increased insulin clearance (measured by euglycaemic insulin clamping) by 9% in healthy people and 15% in diabetic patients (155). Chronic combined strength and endurance exercise intervention of 3 sessions/week (total of 270 min/week) lasting 6 months increased the insulin clearance rate by 51% compared with baseline (evaluated using a 5-h OGTT) in obese adults aged ~70 years, whereas no change in insulin clearance rate was observed in the non-exercise group (156, 157). However, in pre-diabetic people aged ~50 years, 12 weeks of combined aerobic and resistance exercise training of 3 sessions/week (total of 225 min/week) increased insulin clearance by 20.5% (evaluated using hyperinsulinaemic–euglycaemic clamping with stable isotope tracers) only when combined with pharmaceutical intervention (metformin) but not after exercise training alone (158). An acute resistance exercise bout also improved glucose concentration during the 4-h OGTT by 14.5%, despite no effects on insulin concentration and clearance in diabetic patients aged ~50 years (159). The differences between results may reflect differences in study populations (healthy, obese, prediabetic or diabetic) or different methods of measurement, modelling and calculation of hepatic insulin extraction, including the use of fasting C-peptide concentration and insulin kinetics, which relies on assumptions that might not be met. The duration of exercise training may also affect the hepatic insulin response, with <3 months of exercise or <225 min/week having no effect on insulin clearance. It is likely that other mechanisms apart from increased glucose uptake, increased insulin clearance and

reduced insulin resistance, such as inflammatory response, are involved in the improvement in glucose regulation after exercise training, especially in older adults.

1.1.2.5 Glucoregulatory mechanisms induced by exercise training in younger adults

In younger adults, exercise training likely improves glucose regulation by improving glucose uptake in muscles. A 12-week exercise intervention in young overweight sedentary people aged 20–23 years increased the expression of proteins involved in glucose transport within skeletal muscles, such as GLUT4 by 26%, serine/threonine protein kinase (AKT2) by 33% and hexokinase 2 by 28% (160). AKT2 regulates important aspects of cellular metabolism, such as insulin-responsive glucose transport (161). For example, insulin activates AKT2 to promote translocation of GLUT4 to the cell surface and allow glucose uptake *in vitro* (161). Activated AKT2 increases hexokinase activity, which can phosphorylate target proteins that affect glucose uptake and metabolism (162). Four weeks of single-leg endurance exercise on a cycle ergometer increased GLUT4 protein content by 36% compared with the untrained leg in young men (163). An 8-week interval exercise intervention increased muscle insulin receptor content by 2-fold and 1.5-fold in the control group (age 29–55) and in people with metabolic syndrome (age 23–54) (164), which suggests that the exercise-induced glucose uptake is due to an increase in insulin sensitivity in muscles, through an increase in insulin receptor content.

Exercise may also increase skeletal muscle glucose uptake through independent vascular response, without involving insulin, such as an increased perfusion rate in skeletal muscles. In healthy young and lean people, an acute bout of one-legged exercise for 2 h increased local muscle tissue perfusion by 58% and fractional glucose extraction and glucose uptake by 61% compared with the control leg (165). Basal muscle blood flow was 64% higher in young athletes than in their sedentary counterparts (166). In the same study, the basal blood flow after

acute exercise was positively associated with glucose disposal during insulin infusion. These results suggest that in younger people, exercise-associated improvements in glucose regulation occur through improvements in vascular factors which promote glucose delivery and uptake. However, these studies on the improvement in vascular effects and glucose delivery with exercise are mostly observed in young people, with a lack of studies available in older people. While the results are inconsistent in older adults, the increase in GLUT4 protein content with exercise was consistently observed in younger adults, suggesting that apart from vascular effects, exercise also increases glucose transporter and uptake in younger adults. It is likely that changes in glucoregulatory pathways after exercise training differ between younger and older adults. Future research is warranted to elucidate the mechanisms, such as FGF21 and adiponectin, that drive the age-associated differences in glucose regulation after exercise. None of the studies to date have investigated the associations of FGF21 with exercise-related improvement in glucose regulation, or with age-related decline in glucoregulatory function.

1.1.3 Arterial stiffness

1.1.3.1 Ageing and arterial stiffness

Arterial stiffness is an independent risk factor for cardiovascular mortality and is implicated in various metabolic diseases, including hypertension, coronary artery disease and diabetes (167-169). Arterial stiffness can be indirectly measured through pulse wave velocity (PWV), which is the most simple, non-invasive, robust, reliable and reproducible method to determine arterial stiffness (170). Carotid-femoral PWV is considered as the ‘gold-standard’ measurement of arterial stiffness and is widely used in epidemiological studies to show that aortic stiffness is predictive of future cardiovascular events (170). In humans and in obese animal models, development of arterial stiffness precedes the development of metabolic disease, and normalization of metabolic disease restores normal arterial stiffness (171, 172). Age is an

independent determinant of arterial stiffness, and its prevalence increases exponentially with age even in healthy populations (173, 174). The prevalence rates of arterial stiffness (175) and metabolic diseases (176) both increase with age, and arterial stiffness is predictive of all-cause mortality among older people (177). Each unit increment in arterial stiffness parameter is associated with an increase in all-cause mortality by 19% in people aged ≥ 70 years (178). During ageing, the wall of large arteries, especially the aorta, thicken and lose elasticity, which increases the PWV. The increased arterial stiffness reduces the buffering function of the arteries near the heart and leads to increments in systolic and pulse pressure (179, 180). The loss of arterial elasticity and decreased absorption of pressure waves with age increase the arterial wave reflection (181). These changes lead to an increase in cardiac afterload and systolic pressure, which impair coronary blood flow and contribute to the increased risk of age-associated chronic diseases such as cardiovascular diseases (180).

1.1.3.2 Mechanisms underlying the age-associated changes in arterial stiffness

The arterial system is made up of large elastic arteries that are rich in elastin and collagen, and smaller peripheral arteries that are more muscular. The intima and media form the innermost two layers of the arterial wall, between the lumen and vessel wall. A monolayer of endothelial cells lines the intima while smooth muscle, collagen and elastin forms the media. Changes in arterial stiffness are due to structural changes in elastin, collagen and smooth muscle content in the vessel wall, as well as functional changes in the endothelium (182). Collagen and elastin contribute to the stability and compliance of the vessel wall, whose content is regulated by a dynamic process of collagen and elastin production and degradation. With advancing age, inflammatory responses lead to degradation of elastin molecules coupled with excessive production and deposition of collagen in the media and a consequential increase in vascular stiffness (182). During ageing, formation and accumulation of advanced glycation end-

products (AGEs), which are molecules derived from glycolysis that cross-link structural proteins, further contribute to arterial stiffness. For example, 1-month administration of AGEs inhibitor in animals reduced age-related left ventricular stiffness by ~40%, coupled with an improvement in cardiac function (183). In humans, administration of AGEs inhibitor for 56 days also improved arterial stiffness by 8% (184). In ageing arteries, these molecular changes in the extracellular matrix result in a 2–3-fold increase in media thickness and associated hypertrophy of the smooth muscle layer in the vasculature, even in healthy people without cardiovascular disease (185, 186).

Endothelial cell signalling and vascular smooth muscle cell tone both influence arterial stiffness. Vascular tone can be impaired by changes in calcium signalling, as well as autocrine and paracrine mediators such as an increase in expression of vasoconstrictor genes such as endothelin (187), an increase in oxidative stress levels (188), as well as a decrease in nitric oxide (NO) activity (189). During ageing, arterial oxidative stress increases in both humans and animals, and is key in linking older age to endothelial dysfunction and the associated risk of cardiovascular diseases (190). The age-associated increase in ROS production affects vascular smooth muscle and endothelial cell growth and apoptosis, and impairs modulation of endothelial function, all of which contribute to arterial stiffness (191). For example, oxidative stress was 1.6–2-fold higher in healthy older men aged 63 years compared with younger men aged 23 years, and the increase in oxidative stress was negatively associated with endothelium-dependent vasodilation in both younger and older men (192). Acute intravenous anti-oxidant administration also improved endothelial function by 53% in healthy elderly men aged 64 years (193). There was no difference between oral or intravenous administration of anti-oxidants on arterial stiffness in healthy adults, regardless of age (194). These findings suggest that oxidative stress plays a role in endothelial function, which may be a key mechanism driving the age-

associated changes in arterial stiffness. In support, clinically healthy middle-aged men had higher circulating concentration of oxidized low-density lipoprotein (oxLDL), a marker for oxidative stress, which predicted future coronary heart disease events and was related to carotid and femoral artery intima-media thickness and plaque occurrence, which promotes arterial stiffness (195-197).

During ageing, the increase in oxidative stress affects endothelial function by reducing NO production, uncoupling NO-producing endothelial NO synthase (eNOS) and decreasing the availability of essential eNOS cofactor tetrahydrobiopterin (BH4) (187, 198, 199). NO protects arteries against ageing and plays a key role in maintaining endothelial function by inducing vascular smooth muscle relaxation and vasodilation, inhibiting platelet adhesion and aggregation, and its anti-oxidant and anti-inflammatory effects (200-202). The bioavailability of NO can be reduced through increased production of ROS by stress hormones such as angiotensin II (203). Stimulation of angiotensin II increases superoxide and ROS production through nicotinamide adenine dinucleotide phosphate oxidase (NADPH) enzymes. The increase in superoxide and ROS production also reacts with NO to form peroxynitrite and other highly reactive species that promote abnormal vascular tone and reduce arterial compliance. Therefore, the mechanisms driving the age-associated changes in arterial stiffness include structural changes due to elastin degradation and collagen deposition, and functional changes, due to the increase in oxidative stress, which impairs endothelial function and suppresses NO production (Figure 1.2).

Figure 1.2 Vascular mechanisms driving age- and exercise-associated changes in arterial stiffness. Ageing causes both structural and functional changes that increases arterial stiffness, while exercise largely causes functional changes that reduce arterial stiffness in humans. Upward arrows indicate an increase and downward arrows indicate a decrease. NO = Nitric oxide, BH4 = Tetrahydrobiopterin, eNOS = endothelial nitric oxide synthase, oxLDL = oxidized low-density lipoprotein.

1.1.3.3 Exercise and arterial stiffness

Lifestyle modifications, including exercise training, are effective in preventing and improving arterial stiffness (32). Compared with sedentary counterparts, habitually active people with greater cardiorespiratory fitness have reduced arterial stiffness (204, 205). Endurance-trained healthy middle-aged and older men (~48 and 67 years old, respectively) had 20–35% higher arterial compliance than their recreationally active and sedentary counterparts (206). Short-term moderate-intensity aerobic exercise intervention of 3–5 sessions per week for 3–12 weeks reversed vascular stiffening by 18–43% in middle-aged healthy sedentary people and people

with T2DM, which suggests that exercise improves arterial stiffness regardless of health status (206-208).

Both habitual and short-term aerobic exercise attenuate the age-associated increase in arterial stiffness and the associated risk of cardiovascular diseases (206, 209). However, 16–52 weeks of exercise training intervention did not improve arterial stiffness in healthy sedentary elderly or healthy young individuals (210, 211). In younger women (~25 years), high-intensity exercise training for 16 weeks improved arterial stiffness significantly by ~5% but moderate intensity exercise had no effect on arterial stiffness (210). In healthy sedentary older men (aged >65 years), moderate intensity exercise for 52 weeks had no effect on arterial stiffness (211). Elderly men and women who exercised for 2–3 sessions per week (>30 min each session) for the past 25 years had similar central and peripheral arterial stiffness as their sedentary counterparts, although carotid stiffness was ~28% lower in lifelong exercisers than sedentary individuals (212). In the same study, 4–7 sessions per week (>30 min each session) was associated with an improvement in carotid stiffness by ~30% and central arterial stiffness and compliance by 18–38%. The effects of exercise on attenuation of arterial stiffness were not consistently observed. These earlier results support the idea that the intensity, frequency and cumulative weekly duration of exercise may influence the ability of exercise to alter arterial stiffness. It is likely that higher intensity exercise performed over ≥ 3 sessions /week is required to attenuate arterial stiffness. While aerobic exercises are recommended for attenuating arterial stiffness and for the prevention and treatment of cardiovascular diseases, the evidence on resistance training is less clear. In pre-hypertensive men and women not on medication, 4 weeks of 3 sessions/week of resistance training increased PWV whereas aerobic exercise training decreased PWV (213). Similarly, in healthy young men, 4 months of resistance training (3 sessions/week) decreased arterial compliance by 19% and increased beta-stiffness

index by 21%, despite no changes in arterial compliance in the control group (214). Compared with sedentary controls, strength trained young male athletes also had 26% lower arterial compliance (215). These studies support that unlike aerobic exercises, resistance exercise may not attenuate arterial stiffness.

1.1.3.4 Mechanisms underlying the exercise-induced improvement in arterial stiffness

Aerobic exercise can reverse the age-associated increase in arterial stiffness through remodelling of the arterial structure. The increase in arterial stiffening in older mice was completely reversed with 10–14 weeks of voluntary wheel running, through reduction in arterial calcification and collagen protein expression in the arteries (216). However, the structural components related to arterial stiffness seem to be unaltered by exercise training in humans. For example, in previously healthy sedentary elderly people aged >65 years, 1 year of endurance exercise training (~200 min/week) reduced left ventricular afterload to the level observed in master athletes despite no effect on intrinsic structural components related to arterial compliance (211). This finding suggests that exercise modulates the functional rather than structural components of arterial stiffness in humans.

Exercise attenuates the functional components of age-associated arterial stiffness by reducing oxidative stress. In healthy sedentary postmenopausal women, arterial compliance improved by 28% to the level observed in endurance-trained women after acute systemic infusion of anti-oxidants, which scavenge ROS (217). Similarly, in two other studies, infusion of anti-oxidants improved endothelial function only in sedentary older people, but not in regularly active older men and postmenopausal women (218, 219). These findings suggest that habitual exercise improves arterial stiffness in humans by reducing oxidative stress, which mediates improvement in vascular endothelial function.

The reduction in oxidative stress induced by regular exercise may also improve arterial stiffness by preserving NO bioavailability, which helps to maintain endothelial function. Compared with sedentary old mice, old mice who ran regularly had increased activation and expression of eNOS in their arteries, which resulted in greater NO production and improved endothelial function (220, 221). Administration of BH4, which reduces eNOS uncoupling and increases NO production, improved endothelial function by 45% in sedentary older adults but had no effect on endothelial function in regularly active older men (222). This finding suggests that habitual exercise preserves BH4 bioactivity and reduces eNOS uncoupling during ageing, which helps to maintain NO-mediated endothelial function and may reduce arterial stiffness. Taken together, these studies suggest that aerobic exercise attenuates age-associated changes in arterial stiffness by reducing oxidative stress, which increases NO bioavailability and improves endothelial function (Figure 1.2). Oxidative stress is likely a central mechanism that drives age-associated increase in arterial stiffness and reduction of oxidative stress contributes to exercise-associated attenuation of arterial stiffness. It is possible that other potential candidates that regulate oxidative stress, such as FGF21 (49, 50), could mediate the protective effects of exercise in preserving arterial stiffness with age. However, the anti-oxidative properties of FGF21 and its influence on arterial stiffness during ageing and exercise is not known in humans and warrants further investigation, especially in healthy humans.

1.1.3.5 Arterial stiffness and lung function

Reduced lung function is a risk factor for cardiovascular morbidity and mortality (223). Precursors for cardiovascular diseases such as arterial stiffness are also associated with poorer lung function, regardless of age, sex or anthropometry (40-44). For example, aortic stiffness was inversely associated with lung function in men aged 30–70 years without coronary heart

disease (41). With each 2.5 unit increase in PWV, a surrogate indicator of arterial stiffness, forced expiratory volume in 1 s decreased by 195 mL and forced vital capacity decreased by 190 mL (41). In a cross-sectional study involving 13,090 participants, the inverse relationship between lung function and arterial stiffness was age-dependent and was found only in people aged more than 40 years, even after adjustment for cardiovascular risk factors, smoking history and lung disease (42). Lung function was also inversely associated with increased arterial media thickness (44), which suggests that poorer lung function may be linked to increased arterial stiffness. A causal relationship between lung function with arterial stiffness and subsequent cardiovascular risk was suggested in longitudinal studies lasting 4–20 years. For example, a reduction in lung function predicted the future development of carotid atherosclerotic plaques and hypertension in both men and women (40, 224). Lung function is also a strong risk factor for increased arterial stiffness in later life, as evidenced in an 18-year follow-up study in men (43). In patients with airflow obstruction and impaired lung function, arterial stiffness was markedly increased compared with age-matched healthy controls (225, 226). Collectively, these studies support the idea of a common pathway between a decline in lung function and the subsequent onset of arterial stiffness and cardiovascular diseases. While the inverse associations between lung function parameters and arterial stiffness parameters have been consistently found in humans, regardless of ethnicity, health status, age, sex and height (41, 227–230), the underlying mechanisms that drive these associations are not known presently.

Mild inflammatory response may be a potential mechanism, as it is independently associated with both an increase in arterial stiffness and a reduction in lung function (223, 231). For example, pro-inflammatory cytokines such as IL-6 and tumour necrosis factor α (TNF- α), increase arterial stiffness through the impairment of subendothelial release of NO and increase

in vasoconstrictor endothelin-1 release, resulting in impairment of endothelial function and vascular tone (232). Pro-inflammatory cytokines and chemokines such as TNF- α and IL-8 also play important roles in the pathogenesis of lung function impairment, through modulating immune cell function and by communicating between the systemic and pulmonary circulation (233, 234). While inflammatory response might be a potential link between arterial stiffness and lung function, it is likely that other mechanisms exist. Although not fully accounted for, factors including body weight, smoking history, hypercholesterolaemia, hypertension and diabetes also affect the relationship between pulmonary function and arterial stiffness (41). The mechanisms linking lung function and arterial stiffness are likely complex and multifaceted, and other potential mechanisms such as the microbiome, especially in the lungs, should be investigated.

1.1.4 The microbiome

1.1.4.1 Ageing and the gut microbiome

The human body is a complex ecosystem that is host to trillions of microbes, which colonize surfaces of the body, especially the gut. The initial colonization occurs during birth in humans, and the core gut microbiome is established within the first 3 years of life. However, the composition of the gut microbiome changes with advancing age and these changes may be linked to the development of, or protection from various age-associated diseases such as T2DM and cardiovascular diseases (235, 236). For example, compared with young healthy controls (28–46 years old), the core gut microbiome of elderly subjects (>65 years old) consisted of Bacteroidetes-dominated phylum (53% in elderly vs. 8–27% in young) (235). As the young people were healthy controls without metabolic diseases, these results suggest that gut microbiome changes with the ageing process rather than with diseases associated with age. The gut microbiome profile also differs between centenarians and adults aged 60–80 years, and the

changes in microbiome function during ageing occur simultaneously with physiological changes in the human host such as frailty (237, 238). In one study, the loss of microbiome diversity and increased *Clostridium* subpopulation (that do not produce butyrate) in the gut was positively associated with increased frailty and T2DM, even after adjusting for age (239-241). In community dwelling ageing individuals, the gut microbiome alpha diversity was positively associated with cognitive health and negatively associated with physical aspects of frailty (242, 243). Changes in the microbiome composition is also associated with the pathogenesis of various age-related metabolic diseases such as T2DM. Colonisation of germ-free animals with gut microbiome isolated from conventionally raised obese donors resulted in increased insulin resistance in recipient mice (244, 245). Faecal microbiome transplantation from lean donors into male patients with metabolic syndrome increased microbial diversity and improved insulin sensitivity in the recipients after 6 weeks, suggesting the crucial role of the gut microbiota in regulating metabolism and disease (246). These results demonstrate the potential “cross-talking” role of the gut microbiome with major organs in regulating disease pathways (245, 247, 248), suggesting that the microbiome composition and diversity in the gut change with age and these changes may contribute to various age-related conditions and diseases such as frailty and metabolic diseases including T2DM.

However, the inter-individual variation in microbiome profiles among community-dwelling elderly people is larger than young healthy adults (~36 years old), possibly due to the larger impact of external factors influencing the microbiome, such as diet, exercise, disease state and antibiotic treatment (249). The impact of these external factors may be more evident in the elderly than in the young adults, as the elderly are immune-physiologically compromised, which may support a stronger microbiome association with health status in the elderly (249). Exercise increases microbial diversity by increasing the percentage of Bacteroidetes and

decreasing Firmicutes phyla independent of diet (236). Exercise counteracts the changes in microbiota induced by a high-fat diet by changing the relative abundances of Firmicutes, Bacteroidetes and Tenericutes phyla in animal models (236). Dietary changes such as reduction in the amount and variety of fibre-containing foods often occur with advancing age (249). In addition to their role in defending against foreign pathogens, the bacteria in the gut perform important functions such as the anaerobic breakdown of dietary fibre and protein to produce short-chain fatty acids (250), which serve as important metabolites and regulators of energy metabolism and exert beneficial effects on body weight, glucose homeostasis and insulin sensitivity (250). Therefore, lower dietary fibre intake during ageing may influence the profile of gut bacteria and decrease microbiome diversity, which in turn, may influence the risks of chronic diseases with age (251). In addition to lifestyle factors, physiological changes during ageing, such as the increase in low-grade inflammation and decline in immune function, are also associated with changes in the gut microbiome (237, 249, 252).

Although the shared environment has a stronger effect on the gut microbiome than genetic effects, the host genetic profile can influence the gut microbiome (253, 254). In mice, genetic variations in the host accounted for 12% of the variation in the gut microbiome; this was a smaller percentage than that for dietary changes, which accounted for 57% of the variation (255). Monozygotic and dizygotic twin pair studies have shown that the human gut microbiome is shared among family members but that individual variations exist because of specific bacterial lineages present in the gut (253, 256). In a large cohort of 1126 twin pairs, microorganisms from the phyla Firmicutes, Actinobacteria, Tenericutes and Euryarchaeota were shown to be more heritable, but those from the highly abundant Bacteroidetes phylum exhibited little heritability (257). In related individuals ($n = 270$), one-third of faecal bacteria taxa were found to be heritable (258). A subset of the gut microbiome seems to be heritable

and to be influenced by host genetics (259). Hence, heritability should be considered when characterising the microbiome and when investigating the mechanistic pathways on ageing or disease pathogenesis. Although age-associated changes in the gut microbiome and their impact on health have been investigated, to my knowledge, no studies have investigated the microbiome at other sites, such as the lung, during ageing.

1.1.4.2 Lung microbiome

Microbes have also been recently discovered in the lower airways of healthy lungs, which were traditionally considered to be sterile (260). Bacterial communities in the upper respiratory tract (URT) were thought to prevent respiratory pathogens from establishing an infection on mucosal surfaces and spreading to the lower respiratory tract (LRT) (261). The microbiome in the gut has been studied more extensively than that in the lungs in humans mainly because of the need for invasive access to lung samples. Although there are similarities in mucosa-lined luminal surfaces between the lungs and gut, the lungs are exposed to the bidirectional movement of air, mucus and microorganisms with minimal physical barriers (260). Moreover, the gut environment is anaerobic and has a similar temperature throughout, whereas the lungs are aerobic and exhibit a temperature gradient between the upper and lower airways (260). It is possible that these differences affect the establishment of microbial communities so that the microbiome in the lungs differs from that in the gut. A poor local host mucosal immune response in the URT might dysregulate the trafficking of pathogens, leading to recurrent colonization and consequently infection. It is likely that the formation of respiratory microbiota has a role in the maturation of lung architecture and in shaping local immunity (262). The absence of microbial colonization during pubertal maturation results in sustained susceptibility to allergic airway inflammation in adulthood (263). Hence, formation of the lung microbiota induces regulatory cells early in life, which can determine an individual's respiratory health or

disease later in life. It is integral to establish the physiological changes, such as ageing, that may influence the respiratory microbiota, and its role in the maintenance of health in humans.

The constitution of the lung microbiome is determined by microbial immigration, elimination and reproduction rate of the bacterial community. Next-generation sequencing techniques have shown the presence of diverse microbial species in samples from the LRT (264). Bacteria enter the lungs through direct mucosal dispersion and micro-aspiration from the URT, and the oropharynx seems to be the main source of the lung microbiota in healthy adults (265). The symbiotic colonization by the microbiome on the URT mucosal surface prevents overgrowth, inflammation and subsequent local or systemic spread of pathogenic bacteria, including *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* (266). The adult lung microbiome is dominated by Firmicutes (*Streptococcus* spp. and *Veillonella* spp.) and Bacteroidetes (*Prevotella* spp.) (267). The variation in physiological parameters within the lungs, including pH, temperature and oxygen tension, may affect microbial selection and growth. However, spatial microbial diversity within the lungs of healthy individuals is mainly absent (268). These findings suggest that, in healthy people, the lung microbiota comprises a community of low-density and transiently present microorganisms that are derived from the URT, which contrasts with a dominant resident community observed in some disease states (265, 268, 269). Identifying the changes in lung microbiome composition in health and disease will allow for a better understanding on the contribution of the lung microbiome to disease pathogenesis.

1.1.4.3 The lung microbiome in disease states

Acute and chronic lung diseases can alter the immigration, elimination and regional growth conditions in the lung, which may result in different microbial communities between the healthy and in disease states (260). The relationship between the lung microbiota and lung function was investigated using next-generation sequencing techniques that compared samples from healthy people with those from people with chronic obstructive pulmonary disease (COPD) or asthma (39). The lower airways in patients with COPD contained a distinct microbiome that was related to the disease progression and intermittent development of infectious exacerbations. Pathogenic Proteobacteria were found more frequently in the bronchi of adults with asthma or COPD compared with healthy people. By contrast, Bacteroidetes (particularly anaerobic *Prevotella* spp.) were found more common in healthy people than in patients with COPD or asthma (39).

Certain pathogens such as *Pseudomonas*, *Burkholderia*, *Stenotrophomonas* and *Achromobacter* spp. were less prevalent than core microbiota genera in sputum samples from cystic fibrosis patients, but these pathogens have a strong tendency to dominate the bacterial community when present (270). Increasing *Pseudomonas* dominance has been shown to correlate negatively with lung function (270). Given the possibility that normal microbiota modulates the immune responses to pathogens in the nasal mucosa (271), it is likely that the lung microbiome influences the development of some diseases. For example, in healthy individuals, early colonisation of the URT microbiome with high abundance of *Moraxella* and *Corynebacterium* during the first 2 years of life increases the microbiome stability and reduces susceptibility to pulmonary infection (272). This result suggests that the lung microbiome likely affects immunological adaptations of the lungs in early life, potentially shaping inflammatory response and respiratory disease susceptibility later in life. Immune system

dysregulation and inflammatory states are linked to pathogenesis of airway diseases such as COPD (273). For example, macrophages increase by 5–10-fold in the airways of COPD patients, and contribute to disease pathogenesis through the release of inflammatory mediators such as chemokines, cytokines and proteases, and a reduction in the capacity to phagocytose particles (273). However, the exact mechanisms that the lung microbiome affects respiratory disease is not known and warrants further research. Taken together, these studies show that the LRT contains characteristic microbial profiles that differ between the healthy lung with normal function and the diseased lung with restricted airflow and poorer function. These studies also suggest that the lung microbiome composition may play a role in lung health and function, potentially through immunity- and inflammation-related pathways. It is likely that the lung microbiome influences the age-related changes in physiology, which are also driven by immunity and inflammation-associated mechanisms (274). However, studies on the association of the lung microbiome in lung function or in ageing amongst healthy people are lacking, which are important in providing a reference point before disease onset and to provide early prognostic indicators of age-related pulmonary diseases.

1.1.5 Fibroblast Growth Factor 21

1.1.5.1 FGF21 and ageing

FGF21 is synthesized in multiple organs, including liver, brown adipose tissue (BAT), white adipose tissue (WAT), muscle and heart (275). The molecular mechanism of FGF21 signalling is complex and requires co-factors, transmembrane β -klotho (KLB) receptor FGF and receptors (FGFR), to act jointly (275). FGF21 can bind to its receptors and act on multiple target tissues, such as liver, BAT, WAT, and brain in an endocrine, paracrine and autocrine fashion (275). FGF21 has beneficial glucoregulatory, anti-oxidative, and energy-expending properties, which suggest that FGF21 may play a central role in age-associated chronic conditions (276-278). For

example, in the brain, FGF21 stimulates the hypothalamic-pituitary-adrenal axis, increasing sympathetic nerve activity that ultimately increases glucose uptake in the BAT (276). In the liver, FGF21 promotes ketogenesis while inhibiting growth hormone signalling, which results in decreased glucose concentrations (276). FGF21 works as an adipokine by stimulating glucose uptake in both WAT and BAT and increasing WAT browning, which results in increased energy expenditure (276). In the pancreatic islet, FGF21 inhibits growth hormone release and β -cell proliferation while increasing insulin concentrations (276). FGF21 also protects cardiomyocytes from cardiac damage by activating anti-oxidative pathways (279).

In healthy people, circulating FGF21 concentration increases with age from 5 to 80 years (54); serum FGF21 concentration is ~2-fold higher in elderly (>70 years) than in younger (<40 years) people (280). However, decreased serum FGF21 concentration is associated with successful ageing in centenarians (281). The increasing circulating FGF21 concentrations during ageing suggests that FGF21-resistance occurs with age, and FGF21-resistance is associated with the development of age-related-metabolic disease (282, 283). Consistent with this view, lifespan (survival time) was extended by ~36% in transgenic mice with overexpression of FGF21 compared with control mice, which suggests that FGF21 is a pro-longevity hormone (284). This result implies that FGF21 may improve the age-related decline in physiological and metabolic functions, and supports the role of FGF21 as a central mediator of age-associated chronic diseases. Since ageing is associated with chronic conditions such as an increase in oxidative stress-induced arterial stiffness (179) and a decline in glucoregulatory function (285), it is possible that FGF21 may act as a central regulator of these declining health conditions, through the anti-oxidative properties and favourable effects on glucose metabolism. However, few studies have investigated the influence of FGF21 on the age-associated increase in oxidative stress and various chronic conditions such as T2DM, cardiovascular disease and

declining BMD, especially in humans. While FGF21 may be a central mechanism that regulates age-associated changes in physiology, other factors such as exercise, also attenuates age-related physiological functions, such as glucose regulation (143, 144) and arterial stiffness (216). Although the response of FGF21 to exercise training is known (57, 286), studies investigating the associations of FGF21 with the favourable metabolic effects of exercise are lacking.

1.1.5.2 FGF21 and exercise

Acute and short-term moderate and high intensity exercises stimulate the production and increase the basal and post-exercise concentrations of circulating FGF21 in both animals and humans. Acute exercise session of 30 min (50–80% of maximal intensity) in both mice and young male adults (~22 years) led to an increase in circulating FGF21 immediately and up to 1 h after the exercise bout (286). Short-term exercise comprising 5 sessions per week (~15 min each) of multistage incremental exercise (~85% of maximal intensity) for 2 weeks significantly increased basal serum FGF21 concentration by 66% in 60 sedentary women (~24 years) (287). The exercise-induced increase in circulating FGF21 concentration was positively associated with plasma epinephrine and serum free fatty acids (FFA) concentrations. This observation suggests that exercise increases FGF21 concentration through activation of FFA-associated pathways such as peroxisome proliferator-activated receptor α (PPAR- α) and activating transcription factor 4, which increases the liver secretion of FGF21 (288). The liver was suggested to be the main producer of FGF21 during exercise as an acute exercise bout of 30 min in mice increased FGF21 gene expression in the liver, but not in the skeletal muscle or white adipose tissues. The increased hepatic FGF21 gene expression was also coupled with an increase in circulating FGF21 immediately and up to 1 h after the exercise bout, suggesting

that the liver is the main source of increased circulating FGF21 concentration after exercise (286).

However, the increase in FGF21 concentration after exercise has not been consistently reported (289, 290). In younger males and females (~24 years), 3 weeks of sprint interval training (maximal intensity) of ~3 sessions per week decreased plasma FGF21 concentration by 26% (289), while 5 weeks of cycling endurance exercise (60–75% of maximal intensity) of 3 sessions per week (30–45 min each) decreased serum FGF21 concentrations by 12% in older men (62–76 years) (290). Regular exercise training was also reported to decrease serum FGF21 concentration by 17.6% in older men aged ~61 years (57). The difference in FGF21 response to endurance exercise training may reflect differences in the duration, type and intensity of exercise training. It is likely that exercise lasting 3 weeks or more, with at least 3×30 min sessions per week, are required to lower basal FGF21 concentrations. Maximal intensity sprint interval training or moderate intensity endurance exercise likely decrease basal circulating FGF21 concentrations.

The evidence presented suggests that the effects of exercise on FGF21 are likely to depend on the duration of the exercise intervention because short-term (<3 weeks) and long-term (regular and ≥ 3 weeks) exercise interventions induce different FGF21 responses (287, 289, 290). Short-term exercise increases FGF21 concentrations while long-term exercise decreases FGF21 concentrations. This may be explained by the different alterations in body fat and skeletal muscle mass associated with varying exercise duration (291). Acute and short-term exercise training are unlikely to alter visceral fat and lean masses, whereas regular exercise training reduces fat mass, which may decrease circulating FGF21 concentrations through a reduction in FGF21 secretion from adipose tissues (275, 291). Acute exercise stimulates blood flow to

adipose tissues and increases FFA mobilisation (291), which activates PPAR- α pathways and results in stimulation of hepatic FGF21 secretion (288). By contrast, regular exercise training may reduce fat mass and increase FGF21 receptor expression, implying that regular exercise may increase FGF21 sensitivity (292). For example, the decrease in FGF21 concentration after 5 weeks of endurance exercise training was associated with decreased intra-hepatic fat content (290). The reduction in fat mass may stimulate increased FGF21 receptor expression in visceral fat, which may lower circulating FGF21 concentration (292). It is possible that these physiological alterations during exercise can affect FGF21 regulation. However, whether the decrease in circulating FGF21 concentration with long-term exercise training translates to an actual increase in FGF21 sensitivity, and whether FGF21 is associated with the metabolic effects of exercise on health are not known and warrants further investigations.

1.1.5.3 Resistance to FGF21

The paradoxical increase in resting circulating FGF21 concentration in humans during ageing and in people with T2DM may be associated with impaired FGF21 signalling and FGF21 resistance (282, 283). FGF21 resistance is linked to reduced expression of FGFR and KLB receptors in adipose tissues in the insulin-resistant state. In people with T2DM, FGF21 resistance is associated with upregulation of circulating FGF21 concentration along with reduced mRNA expression of FGF21 receptors in adipose tissues and skeletal muscles (293, 294).

Compared with lean and healthy people, people with either T2DM or obesity have increased circulating FGF21 concentration, coupled with a 2–4-fold lower KLB expression in adipose tissues (293). In skeletal muscles, mRNA expression of FGFR1c, FGFR3c and FGFR4 were 2-fold lower in T2DM compared to lean people (293). The higher circulating FGF21

concentration and lower FGFR and KLB receptors in T2DM patients than in healthy lean people suggest that basal FGF21 resistance may reflect the reduced number of FGF21 receptors. This implies that higher circulating concentration of FGF21 and FGF21 resistance observed in T2DM may serve as a compensatory mechanism to offset insulin resistance (283). These results are consistent with the observation of a 2–3-fold progressively higher concentration of circulating FGF21 in obese people, people with impaired glucose tolerance (IGT) and people and T2DM compared with lean healthy people (295, 296). In one study, increased plasma FGF21 concentration in people with IGT or T2DM correlated inversely with peripheral and hepatic insulin sensitivity (295). These results suggest that insulin-resistant states are associated with an impaired insulin and FGF21 response, supporting the view that higher circulating FGF21 concentration is indicative of increased FGF21 resistance, and that FGF21 is likely a protective hepatokine and adipokine that has favourable glucoregulatory and metabolic effects. The increased circulating FGF21 concentration in insulin-resistant states may indicate the presence of FGF21 resistance and reduced gene expression of muscle and adipose tissue FGF21 receptors in humans. However, whether the increased FGF21 concentrations during ageing and in sedentary people are associated with FGF21 resistance is not known and warrants further investigations.

1.1.5.4 FGF21 and glucose regulation

FGF21 plays a central role in improving glucose regulation by increasing glucose uptake in adipose tissues, improving insulin sensitivity, increasing BAT content and energy expenditure, and by reducing hepatic glucose production (297, 298) (Figure 1.3). Chronic administration of exogenous FGF21 improves glucose regulation and insulin sensitivity in obese and diabetic rodents (299) and primates (300), and a single dose of FGF21 decreased glucose area under the curve during the OGTT after 3 days in obese mice (301). This result suggests that FGF21

lowers the glucose response during an acute oral glucose load, implying improved glucose tolerance. FGF21 regulates insulin concentrations in insulin-resistant animal models, possibly through insulin sensitisation, which is proposed to be the main mechanism underlying the glycaemic action of FGF21 (299). In T2DM patients, 4 weeks of exogenous FGF21 administration decreased fasting plasma insulin concentration by up to 40% (302). Insulin infusion also increased FGF21 expression in human skeletal muscle by ~5-fold (303) and increased circulating FGF21 concentration by 1–2-fold in healthy people, obese people, and people with T2DM (293, 303). This finding suggests that the regulatory relationship between FGF21 and insulin is probably independent of metabolic health and could also influence normative changes in physiology with ageing or exercise in pre-disease and healthy individuals.

Figure 1.3 Summary of FGF21 functions on glucose regulation, bone loss, cardioprotective and associated anti-oxidative effects. Upward arrows indicate an increase and downward arrows indicate a decrease.

The mechanisms for FGF21 and insulin cross-talk are unclear, but FGF21 treatment normalized hyperglycaemia in diabetic mice independent of insulin action in the liver and hepatic glucose metabolism (304). Mice with selective hepatic inactivation of insulin receptors (LIRKO) exhibited marked hyperinsulinaemia, which may reflect compensation by pancreatic β -cells to overcome hepatic insulin resistance (304). Despite the absence of insulin signalling in the liver,

FGF21 administration reduced glucose concentration by 40% and insulin concentration by 86% in LIRKO mice (304). These earlier results support the idea that the glucose-lowering effects of FGF21 occur through insulin sensitization and are independent of hepatic insulin. These findings imply that in people with T2DM and insulin resistance, FGF21 may be a therapeutic solution to lower glucose concentrations. However, the role of FGF21 on improving insulin sensitivity and glucose regulation have largely been investigated in animal models or in people with T2DM. It is not known whether FGF21 may be a improve insulin sensitivity and glucose regulation during age-related normative decline in glucose regulation, in healthy people.

1.1.5.5 FGF21 during the OGTT

Circulating FGF21 concentration increases after an acute glucose challenge, which supports the role of FGF21 in regulating glucose metabolism. During an OGTT in humans, plasma FGF21 concentration was lowest at the 60 min time point and then increased to the 120 min time point. A possible explanation for this pattern during the OGTT may be the circadian rhythm of FGF21 concentration in healthy people. FGF21 concentration increases at around midnight, reaches its peak in the early morning hours and declines thereafter (305, 306). Physiological concentrations of FGF21 also modulate circadian behaviour. Compared with wild type mice, transgenic overexpression of FGF21 increased activity during the light phase (where mice normally sleep) by 3-fold, while FGF21 knockout mice had decreased light phase activity by 2-fold (307), suggesting that FGF21 actions affect light-dark cycle activity and exhibit a clear diurnal rhythm. The 24-h profile of circulating FGF21 concentration is opposite to that of serum insulin and blood glucose concentrations. For example, the concentrations of insulin and glucose peak at 30–60 min after meals, at time that corresponds with the lowest serum FGF21 concentration (305). These findings suggest that plasma FGF21 and insulin may be interrelated and contribute to glucose metabolism. It is possible that the increase in FGF21

concentration increases insulin sensitivity, which results in a reduction in insulin concentration during the OGTT. Most OGTTs are measured in 30 min intervals and may not be sensitive enough to identify the changes in FGF21, insulin or glucose concentrations. Future studies that measure glucoregulatory biomarkers at shorter time intervals during the OGTT would be required to understand better the complex interaction and dynamics between FGF21, insulin and glucose concentrations in response to glucose ingestion.

The >60-min delay between glucose ingestion and the increase in circulating FGF21 concentration may reflect a transcriptional response after a glucose load. Endogenous FGF21 production in the liver responds in a dynamic manner during an OGTT, through the activation of the hepatic transcription factor carbohydrate-responsive element-binding protein (ChREBP) in both animals and humans (308). Hepatic ChREBP is a sugar-sensitive transcription factor that increases hepatic FGF21 production and circulating concentration of FGF21 in response to sugar ingestion, through transcription factor PPAR- α -dependent pathways (309, 310). The dynamic response of FGF21 to an acute-bolus glucose intake suggests that FGF21 plays a role in glucose regulation. Most of the research in humans has focused on the therapeutic glucoregulatory roles of FGF21 in disease states, especially in T2DM (276). Much less is known about whether FGF21 has beneficial glycaemic effects in healthy humans and, if so, the response of FGF21 to physiological changes, such as ageing and exercise, that affect glucose regulation.

1.1.5.6 Cardioprotective properties of FGF21

The cardioprotective functions of FGF21 include protecting cardiac cells against hypertrophy, cardiac inflammation and cardiac dysfunction in both *in vivo* and *in vitro* animal models (311) (Figure 1.3). For example, in FGF21-knockout mice, an increase in dilation in the heart was

observed *in vivo* (311). In that study, a greater increase in cardiac hypertrophy and cardiomyocyte volume, through infusion of isoproterenol, are observed in FGF21-knockout mice than in control mice. FGF21 treatment also prevented inflammatory response and cardiac hypertrophy development and increased fatty acid oxidation in the heart, potentially through an increase in PPAR- γ co-activator-1 α expression (311). FGF21 also improves myocardial function by inhibiting cardiomyocyte apoptosis (312) and attenuating pathological cardiac remodelling after myocardial infarction in lean and obese rats (313). A higher FGF21 concentration is positively associated with the incidence of coronary artery disease and all-cause cardiovascular mortality in humans (314, 315). The resting circulating concentration of FGF21 is 1.7–3.1-fold higher in people with cardiovascular diseases including coronary heart disease and atrial fibrillation or in the presence of carotid artery plaque (316–319). Since FGF21 is associated with an improvement in cardioprotective function in animals, the increased basal circulating concentration of FGF21 in people with cardiovascular diseases suggest either a compensatory cardioprotective mechanism of FGF21 to offset cardiovascular disease or FGF21 resistance in diseased states. In patients with T2DM or patients undergoing haemodialysis, FGF21 concentration independently predicted arterial stiffness and was positively associated with carotid intima–media thickness and endothelial dysfunction (320, 321). Collectively, these results support that in diseased populations, FGF21 concentration is positively associated with predictors and risk factors of cardiovascular diseases, such as arterial stiffness. It is plausible that FGF21 also plays a role in mediating the pathogenesis of cardiovascular diseases, by affecting the early risk factors such as arterial stiffness. However, the relationship between FGF21 concentration and arterial stiffness in healthy people is not known presently, and is integral in understanding the contribution of FGF21 and arterial stiffness to subsequent disease progression. Since exercise and ageing influence arterial stiffness, and FGF21 concentrations are independently associated with ageing and exercise, it

is possible that FGF21 is a mediator of the relationship between exercise and ageing with arterial stiffness. The underlying mechanism driving the association between FGF21 and arterial stiffness in diseased people is not known, but could be linked to oxidative stress, which is known to drive arterial stiffness (191).

1.1.5.7 Anti-oxidative properties of FGF21

Protein expression and mRNA of FGF21 increased in cultured cardiac endothelial cells treated with oxLDL, which increases oxidative stress (47), and suggests that FGF21 production responds positively to an increase in oxidative stress. For example, the activation of transcription factor 4 (ATF4) expression, which is induced by oxidative stress, increases hepatic FGF21 expression and circulating serum FGF21 concentration in mice and rats (322). Intracellular stress also directly regulates FGF21 expression and secretion *in vitro*, in a dose- and time-dependent manner (323). The activation of FGF21 signalling *in vitro* in response to intracellular stress occurs through the ATF4-dependent upregulation of the hepatic FGF21 receptor (324). These results imply that oxidative stress increases ATF4 expression, which in turn increases FGF21 expression and concentration, and potentially increases the anti-oxidative effects of FGF21. This finding supports the possibility that FGF21 exerts cardioprotective function and is associated with arterial stiffness through anti-oxidative pathways.

In cell culture models, FGF21 treatment in cardiomyocytes increased the expression of genes involved in anti-oxidative pathways, such as mitochondrial uncoupling proteins and superoxide dismutase, which reduce ROS production (278) (Figure 1.3). FGF21-null mice also had reduced expression of anti-oxidant genes during periods of induced oxidative stress, and replenishment with recombinant FGF21 reversed the oxidative stress observed in FGF21-null mice (278, 325). In animal models, FGF21 administration *in vivo* increases the concentrations

of anti-oxidants and decreases the expression of oxidative stress proteins (49, 50). These findings suggest that FGF21 may play an important anti-oxidative role in preventing the induction of oxidative stress, which is a key driver of arterial stiffness. However, the role of FGF21 in reducing oxidative stress is not well-studied in humans and deserves further investigations. Since ageing, and exercise, influence arterial stiffness through oxidative stress-related mechanisms, FGF21 may mediate the relationships between age- and exercise-associated changes in oxidative stress-induced arterial stiffness.

1.1.5.8 FGF21–adiponectin pathway

Adipose tissues are the major target organ for FGF21 signalling and the insulin-sensitizing effects of FGF21 (326). Adipokines such as adiponectin have cardioprotective effects and regulate glucose and lipid homeostasis through actions within the liver, adipose tissues and pancreas, which attenuate oxidative stress and inflammation by inhibiting NADPH oxidase and nuclear factor kappa-light-chain-enhancer of activated B cells signalling pathways (327, 328) (Figure 1.3). This raises the possibility that FGF21 regulates glucose homeostasis and oxidative stress through the modulation of the secretion of adipokines, including adiponectin. FGF21-deficient mice exhibited lower circulating adiponectin concentration in both obese and lean models (48, 329), whereas transgenic mice with FGF21 overexpression exhibited higher circulating concentration of high molecular weight (HMW) adiponectin (the major bioactive form of adiponectin) (297, 330). A single dose of FGF21 in mice acutely increased total and HMW adiponectin concentrations in a dose-dependent manner (329), and chronic delivery of FGF21 increased the mRNA expression and protein secretion of adiponectin in mouse adipocytes, which led to a twofold increase in serum adiponectin concentrations (48). Collectively, these results support the idea that adiponectin is a downstream effector of FGF21 and its associated glycaemic and cardioprotective benefits (Figure 1.3).

Adiponectin is a cardioprotective, anti-inflammatory and anti-oxidative adipokine (331). After experimentally induced myocardial infarction, adiponectin-null mice receiving FGF21 treatment had a blunted FGF21 response, which resulted in decreased capillary density, increased myocyte apoptosis and increased proinflammatory cytokine expression (332). FGF21-deficient mice administered adiponectin also exhibited alleviated atherosclerotic plaque formation (333). These findings suggest that adiponectin may be a downstream effector of FGF21 and that the FGF21–adiponectin pathway may mediate the cardioprotective and anti-oxidative effects, which could result in improved arterial stiffness. Serum adiponectin concentration increases with age in both healthy individuals and patients with T2DM (55, 334), while exercise increases adiponectin concentrations. For example, both habitual exercise and chronic exercise interventions resulted in 12–97% increase in circulating adiponectin concentration and 54–136% increase in adiponectin receptor expression in skeletal muscles of individuals who were healthy or had T2DM (335–337). Since ageing and exercise affects FGF21 and adiponectin concentrations independently, it is plausible that the FGF21–adiponectin pathway is involved in the age- and exercise-associated metabolic effects.

Some researchers have speculated that insulin sensitization, the main mechanism underlying the glycaemic action of FGF21, may also be mediated by adiponectin (338) (Figure 1.3). Adiponectin is a known insulin-sensitizing adipokine that promotes glucose homeostasis, maintains functional β -cell mass and improves hepatic insulin sensitivity (327, 338). Treatment with exogenous adiponectin in animals lowered glucose concentration and decreased hepatic glucose production in the presence of insulin and in a dose-response manner, suggesting that adiponectin interacts with insulin to improve glucose regulation (339). In humans, genetic adiponectin variants causing higher adiponectin concentrations were found to increase insulin

sensitivity (340). Furthermore, adiponectin- and FGF21-null mice rapidly develop glucose intolerance and are refractory to the insulin-sensitizing effects of a PPAR- γ agonist (341, 342), which suggests that FGF21 and adiponectin can improve insulin sensitivity. Obese adiponectin-knockout mice were resistant to the glucose-regulation benefits of chronic FGF21 administration, including attenuation of hyperglycaemia, insulin resistance, and hepatic steatosis, which were observed in wild-type mice (48, 343).

The effects of FGF21 on adiponectin have also been demonstrated in humans. For example, plasma adiponectin concentration increased in a dose-dependent manner in T2DM patients treated with FGF21 for 28 days (302). In that study, 20-mg dose of exogenous FGF21 administration lowered plasma insulin concentration by 40% from the baseline, which was associated with increased plasma adiponectin concentration and improvement in insulin sensitivity (302). These results suggest that adiponectin may be a downstream effector of the FGF21 pathway and may play an important role in mediating the favourable glycaemic and insulin-sensitizing effects of FGF21 in both animals and humans (302, 329). The association of the FGF21–adiponectin pathway with age- and exercise-related changes in glucose regulation also deserves further investigation.

The exact pathway through which FGF21 stimulates secretion of adiponectin has yet to be elucidated. However, PPAR- γ may be involved in mediating the FGF21-induced secretion of adiponectin. PPAR- γ plays a key role in the transcription and secretion of adiponectin (344, 345), which could modulate the FGF21–adiponectin pathway. In support of this idea, FGF21-null mice were resistant to the insulin-sensitizing effects of adiponectin through PPAR- γ -dependent pathways. Recombinant FGF21 administration restored PPAR- γ expression and

adipocyte differentiation (342). Given that FGF21 increases adiponectin concentrations, it is likely that adiponectin mediates the glycaemic and cardioprotective benefits of FGF21. Although the FGF21–adiponectin axis is a potential therapeutic pathway for the treatment of some metabolic diseases, the associations of the FGF21–adiponectin pathway with glucose tolerance, oxidative stress and arterial stiffness have not been investigated thoroughly in humans. Understanding the FGF21-related pathways on age-associated decline in physiological function will allow for specific therapeutic targets to mitigate or prevent age-related chronic diseases.

1.1.5.9 FGF21 and bone loss

Physiological studies in humans have revealed a negative association between fasting FGF21 concentration and BMD (346, 347). Both pharmacological treatment with FGF21 and FGF21 overexpression in mice resulted in decreased bone mass and severe bone loss, possibly through the inhibition of osteoblast differentiation by PPAR- γ pathways (51) (Figure 1.3). FGF21-knockout mice exhibited decreased bone resorption and increased bone mass and bone formation (51). Pharmacological administration of FGF21 in rats also reduced only trabecular BMD and thickness but not cortical BMD (348). In humans, administration of an FGF21 analogue had adverse effects on bone turnover, as shown by increases in bone resorption markers and decreases bone formation markers (52, 53). However, administration of the FGF21 analogue in non-human primates had no effect on bone mineral content and BMD despite an approximately twofold increase in bone resorption markers (52, 349), suggesting that FGF21 influences bone turnover. These results imply that FGF21 reduces BMD in humans and animals by influencing bone turnover. Despite improving the age-associated decline in glucoregulatory function and decreasing the risk of cardiovascular disease, FGF21 may also

reduce bone mass and cause skeletal fragility during ageing. This finding supports the role of FGF21 as a central mechanism in various age-associated physiological changes.

Specific liver-derived protein insulin-like growth factor binding protein 1 (IGFBP1) is an essential mediator of FGF21-induced bone resorption and bone loss (350) (Figure 1.3). Lower expression of liver IGFBP1 mRNA was observed in FGF21-knockout mice, whereas hepatic IGFBP1 mRNA increased by twofold in FGF21-overexpression mice (350). In IGFBP1-knockout mice, FGF21-induced bone resorption and bone loss were abolished, which suggests that IGFBP1 is a downstream effector of FGF21-induced bone loss (350). In the same study, administration of IGFBP1 increased bone resorption marker concentration by 110% in wild-type mice (350). In humans, growth-restricted twins had higher IGFBP1 concentration and lower bone formation marker concentrations compared with normal weight co-twin controls, which suggests that increased IGFBP1 concentration is associated with decreased bone growth (351).

IGFBP1 promotes bone resorption by binding to its receptor integrin β 1 on osteoclast precursors, which increases RANKL activity and stimulates osteoclastogenesis (51, 350). RANKL-mediated osteoclast differentiation was increased in FGF21-overexpression transgenic mice, as evidenced by greater expression of the mRNA for the osteoclast marker TRAP5b (350). However, IGFBP1 can promote Erk phosphorylation only in the presence of RANKL, and IGFBP1 alone is insufficient to trigger the entire osteoclast differentiation program, which requires the presence of RANKL (350). The current experimental evidence in animals suggest that FGF21-induced bone resorption and bone loss are likely to be mediated through hepatic IGFBP1, which increases RANKL-mediated osteoclast differentiation and bone resorption (Figure 1.3). However, the effects of the FGF21–IGFBP1–RANKL pathway

on bone loss in humans has not been investigated and whether FGF21 is related to the age-associated decline in BMD remains to be elucidated.

1.1.6 Summary

In conclusion, ageing is associated with myriad health conditions such as bone loss, increasing arterial stiffness and decreasing lung function and glucoregulatory function. Exercise habits can help to limit the effects of age-associated changes on the risk of chronic diseases and improve glucose regulation, especially in older adults. Habitual exercise also attenuates the age-associated increase in arterial stiffness through mechanisms such as the reduction in oxidative stress. FGF21 has favourable glucoregulatory, cardioprotective and anti-oxidative properties, although FGF21 administration results in bone loss. Given that ageing and exercise independently influence FGF21 concentration, FGF21 may be a central mediator of the risk of various age-associated non-communicable chronic diseases, raising the potential of FGF21 as a therapeutic target for these age-related diseases. More studies are required to understand the associations of FGF21 with age- and exercise-related changes in physiology, especially in healthy humans (Figure 1.4). The current literature has focused on the microbiome in the gut and its influence on age-associated chronic diseases. However, the associations between the lung microbiome with age-associated changes in lung function and arterial stiffness, especially in healthy humans, remain unexplored.

Figure 1.4. A model showing the relationships between FGF21, the microbiome and exercise and the potential influence on age-associated health conditions. Upward arrows indicate an increase and downward arrows indicate a decrease.

1.2 Rationale for the thesis

Given the glucose-lowering, anti-oxidative and cardioprotective properties of FGF21, it is plausible that FGF21 could influence various age-associated chronic health conditions, such as declining glucoregulatory function, arterial stiffness and low bone mass. However, whether FGF21 is a central mechanism driving these age-associated chronic health conditions is not known, especially during normative ageing in healthy individuals. While exercise is known to attenuate these age-associated chronic health conditions by improving glucose regulation, reducing arterial stiffness and increasing bone mass, the central mechanism to its multipotent effects are not well established. FGF21 is a potential candidate in influencing these favourable effects of exercise, as FGF21 concentrations changes with acute, short-term and habitual exercise (57, 287, 352). Investigating the associated role of FGF21 as a potential central regulator of the effects of exercise and ageing would be crucial for the identification of

therapeutic targets for preventing or decelerating the risk of age-related chronic diseases. This thesis provides insights to the associations of FGF21 with habitual exercise- and age-related effects on glucose tolerance, arterial stiffness and bone mass, in healthy individuals.

In addition, this thesis also seeks to further the understanding of the relationship between ageing health and the lung microbiome. While the effects of ageing on the gut microbiome is fairly well established, none of the studies thus far have investigated the relationship between the lung microbiome and age-associated decline in lung function and health. By comparing the differences in the lung microbiome between elderly and younger healthy individuals, it would allow uncovering of early biomarkers for stratification of at-risk groups toward more refined therapeutic approaches that promote ‘healthy ageing’. It is also important to explore the association of the lung microbiome composition with various physiological changes that occur with ageing, such as the association between the decline in lung function and increase in arterial stiffness. Collectively, these findings may elucidate other mechanisms, such as FGF21 and the lung microbiome, that are associated with the favourable effects of habitual exercise and driving age-associated chronic health conditions.

1.3 Study aims and hypothesis

This thesis comprises four major studies that investigated the associations of FGF21 with age and habitual exercise on glucose regulation, arterial stiffness, and bone mass. One of the studies also explored the effects of age on lung microbiome and the associations with arterial stiffness and lung function in young and elderly family pairs.

1.3.1 Study 1: The FGF21-associated effects on glucose regulation with habitual exercise and ageing

This study investigated the association between FGF21 and glucose regulation, under the influence of exercise habits and ageing in healthy individuals. This study hypothesised that ageing increased FGF21 response while habitual exercise decreased FGF21 response during the oral glucose challenge, and that glucose regulation with habitual exercise and ageing are related to FGF21 pathways. Eighty healthy individuals were recruited and assigned to four equal groups based on age and physical activity profiles i.e., Young Active (YA), Young Sedentary (YS), Older Active (OA) and Older Sedentary (OS). Participants were matched for sex and age in 5-year intervals. Younger adults were ≤ 36 years old and older adults were ≥ 45 years old. Activity groups were classified based on self-declared five-year history of physical activity participation. Health status was clinically documented by a medical doctor through health screening and medical history. Fasted (baseline) blood sample was taken before 75 g of oral glucose solution was ingested, and further blood samples were taken at 30-min intervals for 2 h. Blood glucose, and plasma insulin, FGF21 and adiponectin concentrations during OGTT were measured and compared between groups to determine the age- and exercise-associated impact on glucose regulation.

1.3.2 Study 2: The FGF21-associated effects on arterial stiffness with habitual exercise and ageing

This study investigated the FGF21 pathways that are associated with arterial stiffness, under the influence of ageing and habitual exercise in healthy individuals. The associations of FGF21 with oxidative stress and arterial stiffness were also explored. This study hypothesised that ageing is associated with an increase in oxidative stress and arterial stiffness, and that habitual exercise decreases oxidative stress and arterial stiffness, through FGF21-related pathways.

Eighty healthy individuals were recruited and assigned to four equal groups (YA, YS, OA, OS, mentioned above). Younger adults were ≤ 36 years old and older adults were ≥ 45 years old. Activity groups were classified based on self-declared five-year history of physical activity participation. Health status was clinically documented by a medical doctor through health screening and medical history. Fasted blood sample was taken at baseline and plasma concentrations of FGF21, adiponectin and oxidized low-density lipoprotein (indicative of oxidative stress) were measured. Arterial stiffness was measured indirectly using pulse wave velocity and augmentation index, through the use of applanation tonometry at the carotid pulse and femoral artery. Age- and exercise-associated effects of FGF21 on oxidative stress marker and arterial stiffness parameters were determined by between group comparisons and correlations.

1.3.3 Study 3: The FGF21-associated effects on age-related decline in BMD

This study compared the associations of the FGF21 pathway with bone turnover and differences in BMD between genetically linked young and older family pairs. This study hypothesised that FGF21 concentration, bone turnover markers and bone loss are increased in older than in younger adults, and the FGF21–IGFBP1 regulatory axis is involved in age-associated decline in BMD. Twenty genetically linked healthy family pairs (parent and child) were recruited. Younger adults were 22–39 years old and older adults were 60–81 years old. Participants had their blood samples drawn and serum concentrations of FGF21, IGFBP1, osteoclast marker receptor activator of nuclear factor kappa-B ligand (RANKL), bone formation marker bone-specific alkaline phosphatase (BAP) and bone resorption marker tartrate-resistant acid phosphatase 5b (TRAP5b) were measured and compared between younger and older linked family pairs. BMD was measured using dual-energy X-ray

absorptiometry and measurements were compared between younger and older groups, to determine age-associated effects of FGF21–IGFBP1 pathway on bone turnover and bone loss.

1.3.4 Study 4: Associations of lung microbiome with age-related changes in lung function and arterial stiffness

This study explored the age-associated changes in lung microbiome and their links with arterial stiffness and lung function in younger and older family pairs. This study hypothesised that ageing is associated with changes in the lung microbiome composition, which is associated with age-related changes in lung function and arterial stiffness. Twenty-four genetically linked healthy family pairs (parent and child) were recruited. Younger adults were 22–39 years old and older adults were 60–71 years old. Sputum samples were collected, and DNA was extracted from the samples for targeted amplicon sequencing on the 16s rRNA region to determine the bacterial microbiome composition by metagenomics tools. Arterial stiffness was measured non-invasively and lung function was determined by spirometry. The lung microbiome composition was correlated with measures of arterial stiffness and lung function, and compared between younger and older family pairs, to determine the interaction between age-associated changes in lung function, arterial stiffness and the lung microbiome.

1.4 Structure of the thesis

The thesis has seven chapters as described below:

Chapter 1: Introduction and Literature review

Chapter 2: Study 1. The FGF21-associated effects on glucose regulation with habitual exercise and ageing

Chapter 3: Study 2. The FGF21-associated effects on arterial stiffness with habitual exercise and ageing

Chapter 4: Study 3. The FGF21-associated effects on age-related decline in BMD

Chapter 5: Study 4. Associations of lung microbiome with age-related changes in lung function and arterial stiffness

Chapter 6: Summary and conclusion

Chapter 2: The FGF21-associated effects on glucose regulation with habitual exercise and ageing

2.1 Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disease that affects more than 373 million people worldwide. T2DM is especially prevalent in individuals ≥ 45 years old (9) and is characterised by insulin deficiency caused by beta-cell dysfunction (353). Sedentary behaviour is consistently associated with increased risk of T2DM (354) and physical activity reduces the incidence of T2DM (135, 136). The mechanisms through which exercise improves glucose tolerance and insulin sensitivity are subject of intense research, mostly involving an intricate signalling network that includes improvements in glucose delivery, transport and metabolism (355).

Recently, the role of fibroblast growth factor 21 (FGF21) has been elucidated in human diseases, including T2DM. FGF21 is an endocrine hormone produced mainly by the liver and adipose tissues. FGF21 decreases glucose concentration, improves insulin sensitivity, and reduces hepatic glucose production (297, 298). The insulin sensitizing and metabolic effects of FGF21 have been proposed to be mediated by adiponectin release (338). Despite the favourable glycaemic effects of FGF21, circulating FGF21 concentration in resting humans was paradoxically increased in insulin-resistant states, suggesting the presence of impaired FGF21 signalling and resistance (282, 283). In individuals with T2DM, higher circulating FGF21 is associated with a 2–4-fold lower FGF21 receptor expression in adipose tissue and skeletal muscle, suggesting a compensatory response to offset peripheral or hepatic insulin resistance (283, 293, 294). Compared to healthy individuals with normal glucose tolerance (NGT), the

concentration of circulating FGF21 increases progressively in people who are obese or patients with impaired glucose tolerance (IGT) and T2DM (295, 296). Taken together, these findings suggest that T2DM is associated with the presence of basal FGF21 resistance and increased circulating FGF21 concentration.

Given that both exercise and FGF21 influence glucose homeostasis, there is an interest in understanding the interaction between these two metabolic regulators on glucose regulation. Acute and short-term exercise interventions in both mice and humans are positively associated with increasing circulating FGF21 concentration (286, 287). However, habitual exercise training is associated with 17.6% lower basal FGF21 concentration (57). Similarly, exercise facilitates adiponectin action by upregulating adiponectin receptors and improving glucose uptake (356, 357). For example, long term exercise interventions resulted in 12–97% increase in circulating adiponectin concentration and 54–136% increase in adiponectin receptor expression in skeletal muscles of individuals who were healthy or had IGT or T2DM (335–337). These results imply that FGF21 and adiponectin could mediate the beneficial effects of habitual exercise on glucose regulation.

Ageing is an independent risk factor for glucose intolerance (10). Decrease in glucose uptake with age is associated with higher visceral fat and lower lean masses, and increased insulin resistance (124, 126, 149). Compared with sedentary participants, elderly T2DM patients who performed 6 months of exercise had lower glucose and insulin concentrations by 24–32% during an OGTT (143). Exercise is effective in improving glucose tolerance in both younger and older age groups (143, 145, 146). However, whether FGF21 and glucose tolerance are influenced by habitual exercise and ageing within a healthy population is not known. Therefore, the aim of this study was to investigate the associations of habitual exercise and age with

FGF21 and adiponectin responses during an oral glucose challenge. I hypothesised that ageing increased FGF21 response while habitual exercise decreased FGF21 response during the oral glucose challenge, and that glucose regulation with habitual exercise and ageing are associated with FGF21 pathways.

2.2 Methods

2.2.1 Research participants

Eighty participants were recruited and assigned to four groups based on age and physical activity profiles i.e., Young Active (YA, $n=20$), Young Sedentary (YS, $n=20$), Older Active (OA, $n=20$), and Older Sedentary (OS, $n=20$) participants. The first eighty participants recruited who met the inclusion criteria for the respective age and activity groups were selected for this study. The participants were matched for sex and age, in 5-year age bands, between the active and sedentary groups, within each age group. Matching the participants by age in 5-year age-bands ensured an equal distribution of the age spectrum between 18–36 years old for the younger group and 45–80 years old for the older group. Males and females were equally represented within the groups. The YS and YA participants were 18–36 years old and OA and OS participants were 45–80 years old. Participants were recruited if they were non-smokers, non-habitual alcohol drinkers, not on oral contraceptives or hormone replacement therapy (for females), and their state of health was clinically assessed and documented to be “healthy” (free from disease, medication and treatment) through medical screening by an appointed clinical service provider (Raffles Medical Group, Singapore). None of the participants recruited were obese, or in a high-risk category of body mass index (BMI) for Asians, as their BMI were ≤ 27.5 kg/m². The medical screening assessed for family history of diseases, blood pressure, height, weight, vision, urinalysis, as well as physical examination at the head, neck, nervous system, heart, lungs, abdomen and musculoskeletal sites. The OA group underwent an exercise

electrocardiography stress test to exclude cardiac risk. Physical activity profiles of the participants were based on self-declared five-year history of physical activity participation (type, duration, frequency and intensity). The YS and OS participants engaged in ≤ 2 sessions/week of light intensity aerobic exercises, lasting ≤ 30 min/session on most weeks. The YA and OA groups participated in ≥ 3 sessions/week of moderate to vigorous intensity aerobic exercises for ≥ 45 -min/session on most weeks, which was within the recommended exercise guidelines of 75–150 min/week for moderate-to-vigorous intensity exercise (23). Intensity of exercise was based on self-estimation, with descriptor definitions given to the participants. Light intensity exercises were described as activities causing a slight increase in breathing or heart rate, with participants being able to talk comfortably while doing the activity. Moderate to vigorous intensity exercises were described as activities requiring moderate to hard physical effort, causing larger increases in breathing or heart rate, with participants being unable to talk comfortably while doing the activity. The participants were recruited through posters, social media, and community outreach events. All the participants were briefed on the nature and risks involved in the study and their rights to withdraw their participation without obligation before giving their written informed consent to participate in the study. The procedures in the study were approved by the institutional review board of Nanyang Technological University (IRB-2015-05-029).

2.2.2 Study Design

The participants abstained from caffeine and dietary supplementation and kept to their regular diet and sleep routines for 24 h and refrained from physical exercise for 48 h before their visit to the laboratory. On the day of the experiment, the participants arrived between 0800 h and 0930 h after an overnight fast, declared their state of wellness and had their blood pressure (BP) measured. Nude body weight was measured using an electronic scale (SECA, Hamburg,

Germany) and height was measured using a stadiometer (SECA, Hamburg, Germany). Body mass index (BMI) was calculated as body weight (kg) divided by height (m) squared. Waist circumference (WC) was recorded using a tape measure (SECA, Hamburg, Germany) placed snugly at the waistline, midway between the lowest ribs and iliac crest in a standing position (358), recorded to the nearest 0.1 cm in triplicate and averaged from the measurements.

2.2.3 Blood sampling and analysis

The oral glucose tolerance test (OGTT) involved five intervals of blood sampling with a catheter cannulated into a forearm vein by a qualified phlebotomist. A baseline fasted blood sample of 13 mL was taken prior to the ingestion of a 75-g glucose drink (Trutol 75, ThermoFisher Scientific, United States). Participants remained in a rested condition in the laboratory with additional blood samples (13 mL each) taken at 30, 60, 90 and 120 min after ingestion of the glucose solution.

All blood samples were collected in chilled serum and dipotassium ethylenediaminetetraacetic acid (K2 EDTA) vacutainer tubes. Serum tubes were left at room temperature to clot for 15–30 min, while K2 EDTA tubes were placed in 4 °C immediately after collection. Fasted serum (4 mL) were analysed for triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) (Siemens ADVIA 1800 and ADVIA Chemistry XPT) by a licensed pathology laboratory (Quest Laboratories, Singapore). Glucose concentration was measured immediately after each blood sampling procedure using a handheld glucometer (Accu-Chek, Roche). The remaining blood samples from K2 EDTA and serum tubes were then centrifuged at 1400 x g at 4°C for 15 min. Plasma and serum aliquots (120 µL) were then collected in microcentrifuge tubes and stored at –80°C until analysis.

2.2.4 Bioassays

2.2.4.1 Plasma FGF21 concentration

Fasted human plasma FGF21 concentrations were measured using commercially available sandwich enzyme-linked immunosorbent assay (ELISA)-based assays according to the instructions of the manufacturer (DF2100; R&D Systems, Inc., Minnesota, USA). Briefly, human FGF21 standard was reconstituted from the lyophilized form with deionized water and left for 15 minutes with gentle agitation before serial dilution using calibrator diluent (diluted 1:2). The stock was diluted to assay standards of 2000, 1000, 500, 250, 125, 62.5 and 31.3 pg/mL in duplicates. The calibrator diluent served as the background (blank). Samples were thawed and vortexed briefly and the assay diluent (100 μ L) was added to each well in the 96-well microtiter plate before the adding 50 μ L of diluted 1:2 calibrator diluent (background), standards and undiluted plasma samples to the designated wells in duplicates. Plate was sealed, covered and incubated at room temperature on bench for 2 h. The wells were then emptied through aspiration and the plate was washed four times with the diluted wash buffer, inverting the plate after each wash. The enzyme conjugate (200 μ L, supplied) was then added into each well after the final wash and the plate was sealed and covered with aluminium foil and incubated on bench at room temperature for 2 h. After incubation, the wash step was repeated four times before adding the substrate solution (200 μ L, supplied). The plate was then incubated again for 30 min at room temperature, protected from light. Thereafter, 50 μ L of stop solution was added to each well with gentle tapping on the plate to ensure thorough mixing before reading of optical densities (OD) on the microplate reader at 450 nm and 540 nm (Synergy H1, BioTek, Winooski, Vermont, USA). The concentrations of FGF21 were calculated by matching the OD of the samples against a regression equation established with the standard curve. The standard curve and sample concentrations was generated by subtracting

the OD readings at 540 nm from the OD readings at 450 nm. The corresponding standard and sample wells were then corrected for background by subtracting the OD values of control (blank) wells. A best fit linear curve was then plotted after applying a log transformation of both OD and concentration readings for assay standards, and sample concentrations were calculated using back transformation.

2.2.4.2 Plasma Insulin concentration

Human insulin concentration was measured using commercially available sandwich ELISA-based assays according to manufacturer instructions (Mercodia Insulin ELISA). Briefly, plasma samples at 30 min (OGTT) were diluted 1:2 with calibrator 0 reagent (supplied), which also served as background. Plasma insulin concentration at 30 min for some samples were found to be outside the range of the highest standard provided. The plasma samples at the remaining OGTT time-points 0, 60, 90 and 120 min were analysed undiluted. Standards, calibrator 0 (negative control), samples and controls (25 μ L) were added into each well of the 96-well microtiter plate in duplicates, followed by adding 100 μ L of enzyme conjugate. The microtiter plate was then incubated on a plate shaker at room temperature (700 rpm) for 1 h. The solution in the wells was then decanted and manually washed six times by pipetting 350 μ L of wash buffer into each well during each wash. Thereafter, 200 μ L of substrate solution and 50 μ L of stop solution were added into each well. The plate was then placed on a shaker at 700 rpm for 5 s to ensure proper mixing before measuring the OD at 450 nm with a microplate reader (Synergy H1, BioTek, Winooski, Vermont, USA). Plasma insulin concentration was determined by matching the OD of samples against a cubic spline regression equation established with the standard curve, as per manufacturer's instructions without subtracting the background from both samples and standards.

2.2.4.3 Plasma adiponectin concentration

Plasma adiponectin concentrations were measured using the Adiponectin Human ProcartaPlex Simplex Kit according to the manufacturer's instructions (eBioscience, Affymetrix, Vienna, Austria). Briefly, the frozen plasma samples were thawed on ice and vortex before 500-fold serial dilution with a universal assay buffer. The standards were reconstituted using the universal assay buffer, which was vortexed for 10 s and centrifuged at $2000 \times g$ for 20 s before incubating on ice for 10 min. The standards were serially diluted to concentrations of 194600, 48650, 12163, 3041, 760, 190, 47.5 pg/mL. The magnetic beads were diluted 50-fold with diluted wash buffer and the detection antibody was diluted 50X with antibody diluent. The diluted magnetic beads (50 μ L) were added to each well and placed on a magnetic plate for 2 min to allow the beads to settle at the bottom of each well. The solution in the wells were then removed by quick inversion of the handheld magnetic plate holder and 96-well plate assembly over the sink. With the 96-well plate fixed on the magnetic plate holder, the wash buffer (150 μ L) was then added to each well and the beads were allowed to accumulate at the bottom of each well for 30 s before removing the wash buffer. Standards, samples and universal assay buffer (background) (25 μ L) were then added to their designated wells and sealed with adhesive sheet and covered with opaque microplate lid (supplied). The 96-well microplate was then detached from the magnetic plate holder and incubated on a plate shaker at 500 rpm for 120 min at room temperature. The plate was then washed twice before adding the 25 μ L of detection antibody mixture to each well, followed by incubation on a plate shaker at 500 rpm for 30 min at room temperature. Streptavidin, R-Phycoerythrin Conjugate (SAPE, 50 μ L) was then added to each well after washing the plate twice. Thereafter, the plate was incubated at room temperature for 30 min on a plate shaker at 500 rpm. Reading buffer (120 μ L) was then added to each well after repeating the washing step twice. The plate was then left on the plate shaker at room temperature for 5 min at 500 rpm before reading the fluorescence with the Bio-Plex

200 System (Bio-Rad Laboratories, Inc, CA, USA). Adiponectin concentration was analysed with the Bio-Plex Manager Software by establishing a five-parameter logistic standard curve. All standards and plasma samples for FGF21 and insulin were fresh aliquots without any freeze-thaw cycles, whilst adiponectin plasma samples were freeze-thawed once. All standards, samples and blanks were measured in duplicate, and samples were re-assayed when there was >15% variation from fresh aliquots. All samples had an inter- and intra-assay variation of <15%.

2.2.5 OGTT derived indices

The OGTT area under the concentration versus time curve (AUC) values for glucose, insulin, FGF21 and adiponectin were calculated and analysed using the trapezoidal method (GraphPad Prism, version 7.0, San Diego, CA). Homeostatic model assessment (HOMA) was used to estimate insulin resistance (HOMA-IR) in the fasted state(359), calculated using the formula $[(\text{fasting insulin } (\mu\text{U/ml}) \times \text{fasting glucose (mmol/l)})/22.5]$ (359, 360). Another surrogate measure for insulin sensitivity is the Matsuda index (MI), which is calculated using the formula $(10,000/\text{square root of } [\text{fasting glucose} \times \text{fasting insulin} \times \text{mean glucose} \times \text{mean insulin during OGTT}])$ (361). MI is highly correlated with the rate of whole-body glucose disposal during the euglycemic insulin clamp (361).

2.2.6 Statistics

All statistical analyses were performed using Statistical Package for Social Sciences, version 23 (SPSS, Inc., Chicago, IL). A sample size calculation before the study indicated that 80 participants (20 per group) was needed to have 80% of statistical power for a two-tail hypothesis test at an α level of 0.05, with an effect size of 0.4 (54, 57) (G*Power, version 3.1, Germany). Numerical variables are presented as mean (standard deviation, SD) in text and

figures unless otherwise stated. The participant characteristics and OGTT derived indices were analysed using two-way analysis of variance (ANOVA), with age, activity and age x activity as fixed effects, to assess potential differences between physical activity and age groups. Pearson's correlation was used to evaluate associations between two variables. OGTT-related biochemical parameters measured across time (glucose, insulin, FGF21 and adiponectin) were analysed using a mixed effect model with age, activity, time, and their interactions as fixed effects and subject as random effect. For subgroup analyses within younger and older groups, for differences between activity groups, a similar mixed effects model, was used for glucose, insulin, FGF21 and adiponectin concentrations during the OGTT. A linear regression model was used to compare the relationship between fasted FGF21 concentration and OGTT derived indices in the older group relative to younger group, with adjustment for activity status. Bonferroni adjustment was used for comparisons of time-points during OGTT between groups. Unless otherwise stated, adjustments for BMI and WC were analysed using ANOVA, with BMI and WC treated as continuous predictor variables. Adjusting for sex in the analyses, by including it as a categorical control variable, in the ANOVA did not affect any of the results presented. Hence the data were presented with sex excluded from the model. Due to the skewed distribution of data, logarithmic transformation was performed for glucose, insulin, FGF21 concentrations, and OGTT derived indices. The data for adiponectin concentration was transformed using the cube-root function (362). All the results in the tables and figures are presented using the untransformed data. A $p < 0.05$ was considered statistically significant.

2.3 Results

2.3.1 Study sample and analyses

The data for one participant was excluded from the analyses due to abnormally high plasma FGF21 concentrations i.e., 6-fold higher than the range commonly reported in individuals of

similar activity status. As such, the data were analysed on 79 participants i.e., n=19 YA, n=20 YS, n=20 OA and n=20 OS. The demographics of the participants are presented in Table 2.1. Age and sex were equally and evenly distributed between active and sedentary participants within younger and older groups (Table 2.1).

Table 2.1 Mean (SD) of participant demographics, exercise history, anthropometry, blood pressure, lipids and OGTT derived indices

	Young Sedentary (YS)	Young Active (YA)	Older Sedentary (OS)	Older Active (OA)	Age	Act	Age × Act
<i>n</i>	20	19	20	20			
Sex <i>n</i> (M/F)	10/10	10/9	10/10	10/10			
Exercise Intensity	Light	Mod–High	Light	Mod–High			
Exercise (min/week)	18 (27)	410 (177)	17 (28)	373 (230)	0.560	<0.001	0.596
Age (years)	27 (5)	27 (5)	56 (8)	56 (9)	<0.001	0.666	0.875
Weight (kg)	59.3 (7.3)	59.0 (10.6)	63.3 (13.5)	60.7 (9.3)	0.240	0.497	0.930
Height (cm)	169.6 (8.2)	166.1 (9.3)	163.3 (8.9)	166.4 (8.5)	0.125	0.948	0.085
BMI (kg/m ²)	20.6 (2.2)	21.2 (2.0)	23.6 (3.9)	21.8 (1.5)	0.001	0.524	0.051
WC (cm)	73.0 (5)	72.4 (6.5)	81.2 (8.4)	76.5 (6.2)	<0.001	0.083	0.218
BP (systolic) (mmHg)	110 (9)	109 (5)	117 (11)	114 (12)	0.005	0.351	0.681
BP (diastolic) (mmHg)	67 (8)	63 (5)	75 (8)	69 (8)	<0.001	0.002	0.599
TC (mmol/l)	5.0 (0.7)	4.9 (1.0)	5.6 (0.7)	5.8 (0.9)	<0.001	0.595	0.385
HDL-C (mmol/l)	1.6 (0.3)	1.9 (0.5)	1.6 (0.5)	2.0 (0.5)	0.983	0.003	0.792
LDL-C (mmol/l)	3.0 (0.6)	2.6 (0.7)	3.3 (0.6)	3.4 (0.7)	<0.001	0.430	0.194
TG (mmol/l)	0.8 (0.2)	0.7 (0.2)	1.2 (0.4)	0.9 (0.4)	<0.001	0.003	0.521
Fasted Glucose (mmol/l)	4.8 (0.3)	4.8 (0.2)	5.0 (0.3)	5.0 (0.4)	0.035	0.807	0.550
Fasted Insulin (mU/L)	5.8 (2.5)	4.8 (2.4)	6.7 (4.1)	4.0 (2.0)	0.582	0.008	0.306
Fasted FGF21 (pg/mL)	118 (84)	65 (50)	170 (115)	129 (100)	0.039	<0.001	0.833
Fasted adiponectin (µg/mL)	7.1 (5.0)	6.6 (3.4)	6.4 (3.1)	7.6 (4.3)	0.693	0.594	0.452
OGTT derived indices							

HOMA Index	1.25 (0.58)	1.02 (0.53)	1.49 (0.88)	0.90 (0.45)	0.761	0.006	0.341
Matsuda Index	7.6 (2.8)	11.0 (4.9)	7.3 (5.4)	11.4 (5.9)	0.532	<0.001	0.427
Glucose AUC (mmol• 2 h/L)	12.4 (2.0)	11.3 (1.5)	12.9 (2.0)	12.9 (2.0)	0.014	0.173	0.206
Insulin AUC (mU• 2 h/L)	83.4 (32.6)	58.2 (27.0)	105.8 (55.4)	55.7 (21.5)	0.386	<0.001	0.333
FGF21 AUC (pg• 2 h/mL)	210 (132)	112 (91)	354 (238)	154 (112)	0.015	<0.001	0.948
Adiponectin AUC (µg•2h/mL)	13.3 (9.3)	12.5 (6.7)	12.7 (6.7)	14.8 (8.1)	0.434	0.604	0.453

Body mass index (BMI), waist circumference (WC), systolic and diastolic blood pressure (BP), total cholesterol (TC), High density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), Triglyceride (TG), glucose, insulin, FGF21 and adiponectin area under the concentration versus time curve (AUC) during the OGTT. Mod = Moderate, Act = Activity.

2.3.2 Anthropometry, blood pressure and lipids

Differences in demographics, anthropometry, blood pressure and fasting metabolites are shown in Table 2.1. The older adults had higher BMI and WC ($p = 0.001$ and $p < 0.001$) than the younger adults, independent of exercise habits. Both systolic and diastolic BP increased with age ($p = 0.005$ and $p < 0.001$), and only diastolic BP was lower with habitual exercise ($p = 0.002$). TC, LDL-C and TG concentrations were higher in older than younger adults (all $p < 0.001$), while HDL-C was higher, and TG was lower in active than in sedentary individuals (all $p = 0.003$).

2.3.3 OGTT derived indices

Fasted blood glucose ($p = 0.035$) and plasma FGF21 ($p = 0.039$) concentrations were higher in older than younger adults, independent of exercise habits. However, adjusting for BMI and WC removed age-related differences in fasted blood glucose ($p = 0.108$) and plasma FGF21 ($p = 0.100$) concentrations. Compared with their sedentary counterparts, active individuals had 30% lower fasted plasma insulin concentration ($p = 0.008$) and 46% lower fasted plasma

FGF21 concentration, independent of age ($p < 0.001$) (Table 2.1). Active individuals were also 55% more insulin sensitive ($p < 0.001$) and 29% less insulin resistant ($p = 0.006$) than sedentary individuals on average, independent of age. There was no effect of age on insulin sensitivity ($p = 0.532$) and insulin resistance ($p = 0.761$) (Table 2.1).

Independent of age and exercise habits, fasted plasma FGF21 concentration was positively associated with fasted plasma insulin ($r = 0.383$, $p < 0.001$; Figure 2.1A) but not fasted blood glucose ($r = 0.202$, $p = 0.074$) concentrations. No association between fasted blood glucose and plasma insulin was observed, regardless of age and exercise habits ($r = 0.104$, $p = 0.360$). Conversely, glucose AUC was positively associated to insulin AUC when analysed as an entire cohort ($r = 0.355$, $p = 0.001$; Figure 2.1B). Likewise, FGF21 AUC was also positively associated with insulin AUC ($r = 0.427$, $p < 0.001$; Figure 2.1C) but not with glucose AUC ($r = 0.160$, $p = 0.160$), regardless of age and activity status.

Figure 2.1 Pearson's Correlation between fasted plasma insulin and FGF21 concentrations (A) Glucose and insulin area under the curve (AUC) (B) FGF21 and Insulin AUC (C) in response to 75g oral glucose load.

2.3.4 Glucose and insulin concentrations during OGTT

Blood glucose concentration during the OGTT was not different between active and sedentary individuals, independent of age (Activity $p = 0.115$; Activity x Time $p = 0.213$) (Figure 2.2A). Glucose response was approximately 9% higher in older than younger individuals ($p = 0.008$) across all OGTT time points, independent of activity status ($p = 0.458$) (Figure 2.3A). However, adjusting for BMI and WC attenuated the difference in blood glucose response during the OGTT between younger and older groups ($p = 0.057$).

Compared with sedentary individuals, active individuals had an approximately 40% lower post-prandial insulin concentration during the OGTT, independent of age (Activity $p < 0.001$; Activity x Time $p = 0.056$) (Figure 2.2B). In contrast, insulin concentration did not differ between younger and older adults (Age $p = 0.527$; Age x Time $p = 0.250$) regardless of exercise habits, even after adjusting for BMI and WC (Age $p = 0.748$, Age x Time $p = 0.075$) (Figure 2.3B). There was no interaction effect between age, activity and time for blood glucose and plasma insulin concentrations during the OGTT.

2.3.5 FGF21 concentrations during OGTT

The FGF21 response to the glucose load were 53% lower in active than in sedentary individuals and were different over time, regardless of age (Activity $p < 0.001$; Activity x Time $p = 0.004$) (Figure 2.2C). FGF21 concentration during the OGTT were also approximately 58% higher overall in older than younger participants, regardless of activity status (Age $p = 0.014$; Age x Time $p = 0.057$) (Figure 2.3C). However, adjusting for BMI and WC negated age-related differences in FGF21 response during the OGTT (Age $p = 0.054$; Age x Time $p = 0.120$).

Figure 2.2 Mean (SD) of blood glucose (A), and plasma insulin (B), FGF21 (C) and adiponectin (D) concentrations before and 2 hours after 75g oral glucose load. ††† = $p < 0.001$ for main effect of Physical Activity. ** = $p < 0.01$ for interaction effect of Physical Activity and Time. Black squares represent habitually active participants and open squares represent sedentary participants.

Figure 2.3 Mean (SD) of blood glucose (A) and plasma insulin (B), FGF21 (C) and adiponectin (D) concentrations before and 2 hours after 75g oral glucose load. * = $p < 0.05$, ** = $p < 0.01$ for main effect of Age. Black squares represent younger groups and open squares represent older groups.

2.3.6 Adiponectin concentrations during OGTT

There was no effect of activity ($p = 0.611$) and age ($p = 0.448$) on plasma adiponectin concentration during the OGTT. The mean profile of plasma adiponectin remained constant throughout the OGTT for all groups (Figure 2.2D and 2.3D).

2.3.7 Association between fasted FGF21 concentration and OGTT derived indices

Compared with the younger group, the older group had a stronger negative association between insulin sensitivity (MI) and fasted plasma FGF21 concentration ($p = 0.045$). Activity status had

no effect on the relationship between MI and fasted plasma FGF21 concentration. However, the association of insulin resistance (HOMA-IR) and fasted plasma FGF21 concentrations were not different in younger and older adults ($p = 0.089$).

2.3.8 Association of habitual exercise and OGTT parameters within younger and older groups

In the younger group, blood glucose ($p = 0.023$; Figure 2.4A) and plasma insulin ($p = 0.003$; Figure 2.4C) and FGF21 ($p = 0.003$; Figure 2.4E) concentrations during OGTT were lower in YA than YS individuals. However, only blood glucose ($p = 0.024$) and plasma insulin ($p = 0.046$) concentrations, but not FGF21 ($p = 0.184$) concentration changed differently over time between YA and YS individuals.

In contrast, within the older group, plasma insulin ($p = 0.001$; Figure 2.4D) and FGF21 ($p = 0.002$; Figure 2.4F) concentrations but not blood glucose concentration ($p = 0.983$; Figure 2.4B) during the OGTT were lower in OA than OS individuals. Only plasma FGF21 response to the glucose load was affected by time ($p = 0.011$, Figure 2.4F). Plasma adiponectin concentration during the OGTT was not influenced by the effects of activity in both younger ($p = 0.882$) and older groups ($p = 0.330$) (Figure 2.4G and H). Adjusting for BMI and WC did not influence these results.

Figure 2.4 Mean (SD) on concentrations of blood glucose (A, B) and plasma insulin (C, D), FGF21 (E, F) and adiponectin (G, H) within the younger and older groups before and 2 hours after 75g oral glucose load. † = $p < 0.05$ and †† = $p < 0.01$ for main effect of Physical Activity. * = $p < 0.05$ for interaction effect of Physical Activity and Time. Black squares represent active participants and open squares represent sedentary participants.

2.4 Discussion

The present study is the first to show the association between FGF21 and glucose regulation with ageing and habitual exercise. However, neither age nor habitual exercise status were associated with adiponectin concentrations during the OGTT, suggesting that adiponectin is unlikely involved in the FGF21 glucoregulatory axis. Subgroup analyses comparing the exercise-associated effects on glucose, FGF21 and insulin concentrations during the OGTT, between younger and older adults in the present study, also suggest that the mechanisms driving habitual exercise-related improvements in glucose tolerance may differ between age groups.

Compared with sedentary individuals, habitually active individuals had lower fasted and postprandial plasma FGF21 and insulin concentrations during the OGTT, implying that active individuals require a lower FGF21 response to normalize hyperglycaemia. Although habitually active and sedentary groups had different plasma FGF21 and insulin concentrations during the OGTT, blood glucose and plasma adiponectin concentrations were similar, regardless of age. This study results concurred with a study on middle-aged and elderly Japanese men, which found that fasted serum FGF21 concentration was negatively associated with cardiorespiratory fitness and peak oxygen uptake (57). Five weeks of endurance training in elderly men also reduced fasted serum FGF21 concentration (290). Habitually active individuals also have 25–47% lower fasted insulin concentration and ~2-fold increase in peripheral insulin action compared with sedentary individuals (144, 363). Furthermore, exercise-induced improvements in peripheral insulin action on glucose disposal was reversed after 2–3 days of exercise cessation in young athletes (~30 years old) (363). Similar to my results, 3 days reduction of physical activity from >10,000 steps to <5,000 steps had no effect on the glucose AUC but increased insulin AUC during OGTT in healthy young adults, suggesting that exercise

improves insulin action (364). In that study, the reduction in physical activity also increased glucose concentration during the OGTT at 30 min and 60 min time points (364). In contrast, my study showed no change in glucose concentrations during the OGTT between active and sedentary individuals. The differences could be due to the cross-sectional comparison of habitually (>5 years) sedentary and active individuals in my study, rather than an intervention study to reduce physical activity in that study. My study also included older adults (45–80 years old) but that study included only young healthy adults (20–35 years old). This present study showed a 1.9-fold higher circulating basal plasma FGF21 concentration in sedentary than in habitually active healthy individuals. Similarly, compared to healthy lean individuals, individuals with T2DM and obesity had ~2–2.7-fold higher circulating basal FGF21 concentrations (293, 294). The higher circulating FGF21 concentrations in T2DM patients is indicative of basal FGF21 resistance due to diminished expression of FGF21 receptors or increased peripheral and hepatic insulin resistance (283). These findings could imply that habitual exercise is associated with lower basal FGF21 and insulin resistance. However, in contrast to the present findings, acute and short-term exercise interventions resulted in a 1–3-fold increase in circulating FGF21 concentrations (286, 287, 352). Unlike acute and short-term exercises, long-term exercise training reduces visceral fat mass and increases muscle mass, which could lower FGF21 secretion (57, 291, 292). These results suggest FGF21- and insulin-dependent pathways can potentially contribute to better insulin sensitivity and glucose regulation with habitual exercise.

The favourable glycaemic and insulin-sensitizing effects of FGF21 was shown to be mediated through the effects of adiponectin (48, 302, 329). Adiponectin enhances insulin sensitivity, inhibits hepatic glucose production and maintains functional beta cell mass (327, 340, 365). However, I found that FGF21, but not adiponectin, was influenced independently by both

habitual exercise and age, suggesting that habitual exercise and ageing unlikely influence glycemia through FGF21–adiponectin axis. This study results agree with observations in mice that adiponectin was not upregulated in response to both acute and chronic FGF21 administration, which improved insulin sensitivity in control and adiponectin null-mice (326). In contrast to my results, adiponectin was found to be a downstream effector of FGF21, as FGF21 administration significantly increased circulating concentration of adiponectin by 4-fold in animals and by 1.8-fold in humans (48, 302, 329). The disparity on the FGF21–adiponectin axis (48, 302, 326, 329) could be due to the differences in mouse lines, dose and source of FGF21. For example, studies supporting the FGF21–adiponectin axis used adiponectin knockout mice on high fat diet for 4–6 weeks (48, 329), instead of 12 weeks (326). The dose of FGF21 could also account for the discrepancy observed, as studies supporting the effects of FGF21 on adiponectin administered 0.3–0.5 mg/kg/day of FGF21, compared to 1 mg/kg/day in studies observing no effects. The source of FGF21 used were also different across studies, including pharmacological variant of FGF21, recombinant human FGF21, or recombinant mouse FGF21 (48, 302, 326, 329). Although the FGF21–adiponectin axis was supported in obese humans with T2DM (302), the participants in this study were non-obese and healthy individuals with normal glucose tolerance, which may account in part for the differences observed. Studies supporting the activation of the FGF21–adiponectin axis administered supra-pharmacologic and supra-physiological doses of exogenous FGF21. FGF21 could also regulate glucose independently of adiponectin through direct signalling to the brain, inter-organ crosstalk between the liver and brain, or by increasing energy expenditure and improving glucose uptake in brown adipose tissues (304, 307, 366). Other factors such as pro-inflammatory cytokines and oxidative stress could also regulate adiponectin independently of FGF21. Taken together, FGF21-dependent pathways and glucose regulation are likely

associated with habitual exercise and ageing in healthy humans, but the association with adiponectin as a downstream effector is not supported by this study.

This study findings also suggest that age-associated changes in glucose regulation could be influenced by FGF21. Both blood glucose and plasma FGF21 concentrations during the OGTT were higher in older than in younger individuals, independent of exercise habits. The negative association between fasted plasma FGF21 concentration and insulin sensitivity was also greater in the older than the younger groups, independent of activity status. It is likely that increased plasma FGF21 concentration compensated for the poorer glucose tolerance that occurs with age. In agreement with my results, a negative correlation between insulin sensitivity and serum FGF21 concentration was also found in older adults (>60 years) with NGT, IGT and diabetes (367). My results also concurred with findings from community dwelling older adults (~63 years) showing a positive association between basal circulating FGF21 concentration and abnormal glucose metabolism (367). Circulating FGF21 concentration was also shown to be positively associated with age in healthy individuals (aged 5–80 years), independent of body fat (54). These findings suggest that ageing is linked to higher FGF21 concentrations and lower insulin sensitivity. Therefore, the age-associated increases in plasma FGF21 concentration during the OGTT in this study could alter insulin sensitivity and compensate for the concurrently higher glucose concentrations.

In this study, plasma adiponectin and insulin concentrations during the OGTT were not influenced by age, suggesting that the age-associated effects of FGF21 on glucose tolerance are independent of both insulin and adiponectin. In agreement with my results, age-associated changes in insulin responses to both an oral dextrose tolerance test and a mixed-meal test were

not observed in men aged 47–90 years (368). Younger and older participants also had similar fasted insulin concentration regardless of activity status (144). In contrast, other studies have shown that serum adiponectin concentration increases with age in both healthy individuals and T2DM patients and is negatively associated with insulin resistance (55, 334). These differences could be attributed to the measurement of total adiponectin in the present study, compared to high-molecular-weight adiponectin measured in other studies. These present findings are also limited to a clinically healthy population. It is plausible that FGF21-dependent pathways directly regulate age-associated increases in glucose concentrations during the OGTT, independent of adiponectin.

This study demonstrated for the first time that habitual exercise-associated improvements in glucose regulation may be driven by different mechanisms between younger and older groups. Within the younger group, circulating blood glucose and plasma insulin and FGF21 concentrations during the OGTT were lower in active than in sedentary individuals. In contrast, in the older group, circulating blood glucose concentration during the OGTT was independent of exercise habits, despite lower plasma FGF21 and insulin concentrations in active than in sedentary participants. Therefore, it is likely that the lower post-prandial plasma insulin and FGF21 concentrations resulted in a corresponding decrease in glucose concentrations only in YA compared to YS. Exercise habits could have influenced glycaemic response in the older group by increasing insulin-dependent peripheral glucose uptake and peripheral insulin sensitivity (158, 369, 370). OA and OS individuals could also have similar endogenous glucose production or hepatic and adipose insulin sensitivity (369, 370). In contrast, within the younger group, exercise lowered blood glucose concentration and increases glucose uptake directly through insulin-independent mechanisms, such as greater vascular muscle perfusion rate and glucose transporter upregulation (146, 165, 166, 371, 372). Taken together, this study results

support that habitual exercise-associated improvement in glucoregulatory mechanisms differ between younger and older individuals

A key limitation of the present study is the cross-sectional observational study design and more longitudinal studies are required to adjust for other potential confounders with ageing and exercise. The observed relationships of FGF21 during the OGTT with ageing and habitual exercise are associative and cannot conclude causality of FGF21–adiponectin pathway in driving glucose metabolism. However, based on the discussed mechanisms observed using other animal infusion and knockout models, it seems likely that FGF21–adiponectin axis is a compensatory response to offset and mediate insulin resistance during glucose ingestion, rather than a cause of insulin resistance. Future studies could compare tissue specific expression of FGF21 and its receptors, such as in the adipose tissues or muscle, during an oral glucose challenge intervention in humans. These experiments could also be studied in the context of exercise and ageing, by including a longitudinal arm or an exercise intervention. To account for confounding circadian effects and minimize diurnal variation on the biomarkers measured, all the study participants came in during 8.30 to 9.30 am, albeit on different days of the week. This present study studied the effects of habitual exercise and ageing on glucose tolerance in a healthy population, whether these effects are translatable to a clinical population is not known. Exercise measures were self-reported based on the type, intensity, frequency and duration of regular exercise per week, which could be subjected to recall bias. Future studies using more objective measures of physical activity and exercise should be undertaken. Although physical activity was not objectively measured in the present study, participants were instructed to abstain from exercise 48 h before their visit to the laboratory, to avoid the effects of acute exercise on blood metabolic biochemical markers and prevent inflation of the observed effects.

In conclusion, habitual exercise is associated with lower plasma FGF21 and insulin concentrations both fasted and during an oral glucose challenge, while ageing is associated with higher blood glucose and plasma FGF21 concentrations both fasted and during an oral glucose challenge. Adiponectin unlikely modulates the downstream effects of FGF21 on glucose regulation. These findings suggest that FGF21-related mechanisms may be involved in the age- and habitual exercise-associated effects on glucose regulation. Nonetheless, these findings are preliminary and the precise mechanisms driving the difference in FGF21 and glucose regulation with age and exercise remain to be elucidated.

Chapter 3: The FGF21-associated effects on arterial stiffness with habitual exercise and ageing

3.1 Introduction

Arterial stiffness is an independent risk factor for cardiovascular mortality and is involved in various metabolic diseases (167). With normative ageing, arterial stiffness increases exponentially, even within a healthy population (173). The ageing process independently contributes to the development of arterial stiffness, leading to functional changes in major arteries, including endothelial dysfunction and impaired smooth muscle signalling (179). Age-associated vascular endothelial dysfunction is mainly due to a reduction in the bioavailability of endothelial-derived nitric oxide (NO) (188, 189, 198). In physiological conditions, endothelial NO synthase (eNOS) produces NO, which have vasodilatory and anti-inflammatory characteristics to protect the ageing arteries (187, 198). Endothelial NO also induces vascular smooth muscle relaxation and acts as an antioxidant (200), which inhibits the increase in oxidation of low-density lipoprotein (LDL) caused by increased oxidative stress in the vascular wall (200, 373, 374).

Oxidative stress increases during ageing and is usually caused by an accumulation of free radicals and reactive oxygen species (ROS), coupled with a lack of antioxidants (78-80). Oxidative stress is also involved in the pathophysiology of arterial stiffness and cardiovascular diseases, as it contributes to endothelial dysfunction through uncoupling of eNOS and inactivation of NO (375, 376). Ageing is associated with ~2-fold increase in oxidative stress (192), which directly affects endothelial function and may increase arterial stiffness (193). In clinically healthy middle-aged men, higher circulating concentration of oxidized LDL (oxLDL), a marker for oxidative stress, could predict future coronary heart disease events and

was related to carotid and femoral artery intima-media thickness and plaque occurrence, which promotes arterial stiffness (195-197). Hence, age-associated arterial stiffness is a possible consequence of increased oxidative stress, which may influence endothelial function through reduction in NO bioavailability.

Both habitual and short-term aerobic exercise is effective in preventing and improving arterial stiffness (206, 209). The mechanisms through which exercise improves arterial stiffness include vascular function alterations, which are influenced by smooth muscle tone and endothelial function. Modest amount of exercise habits of 2–3 sessions per week (>30 min each session) improved smooth muscle tone through increasing total arterial compliance, decreasing blood pressure and cardiac afterload (212). Endothelial function also improved with both habitual exercise and short-term (6–12 weeks) exercise interventions in healthy young and older individuals and in patients with cardiovascular disease (79, 377-379).

Fibroblast growth factor 21 (FGF21) is a novel endocrine hormone that is predictive of cardio-metabolic disorders (314, 315), as FGF21 protects against cardiac hypertrophy and dilation and cardiac dysfunction in animal models (311). However, people with cardiovascular diseases have increased resting circulating concentration of FGF21 (318, 319), suggesting a compensatory cardioprotective effect of FGF21 or FGF21 resistance in cardiovascular disease states. FGF21 concentration also independently predicted arterial stiffness and was positively associated with carotid intima-media thickness, endothelial dysfunction and arterial stiffness in haemodialysis and type 2 diabetic patients (320, 321). These results support the relationship between FGF21 and the development of arterial stiffness, potentially through regulating oxidative stress and vascular endothelial function. For example, in animal models, circulating FGF21 concentrations and FGF21 protein and mRNA expression increased with induced

oxidative stress (322) and with oxLDL treatment (47). FGF21 administration to animals *in vivo* increased the concentrations of antioxidants and decreased oxidative stress protein expression and ROS production (49, 50, 278). These results suggest that oxidative stress increased FGF21 expression and concentration, which in turn promotes the anti-oxidative effects of FGF21, potentially influencing arterial stiffness.

FGF21 administration also upregulates adiponectin concentration, which is a cardioprotective, anti-inflammatory adipokine (48, 329). Unlike FGF21, the link between adiponectin and arterial stiffness has been studied extensively (380). Adiponectin concentrations are negatively associated with arterial stiffness (381-383) and plasma adiponectin concentrations independently predicted arterial stiffness progression (384). Adiponectin could influence arterial stiffness through its antioxidant capacity, increasing vasodilation and reducing systemic oxidative stress (328, 385-387). It is possible that the FGF21–adiponectin axis is involved in the regulation of oxidative stress and arterial stiffness. Therefore, this study explored the associations between FGF21–adiponectin pathways and arterial stiffness, under the influence of ageing and habitual exercise in healthy individuals. The associations of FGF21 with oxidative stress and arterial stiffness were also explored. I hypothesised that ageing is associated with an increase in oxidative stress and arterial stiffness in healthy individuals, and that habitual exercise decreases oxidative stress and arterial stiffness, through FGF21- and adiponectin-related pathways.

3.2 Methods

3.2.1 Research participants

The participant criteria for recruitment were described in “Methods” section 2.2.1 in Chapter 2. Some participants recruited in Chapter 2 had insufficient blood samples required for analysis

and were excluded in this study. As such, 7 participants who met the same inclusion criteria described in Chapter 2 for the respective age and activity groups were further recruited for this study, in order to make a total of 80 participants. The procedures in the study were approved by the institutional review board of Nanyang Technological University (IRB-2015-05-029).

3.2.2 Study Design, anthropometry and blood sampling

The study design and anthropometry measurements are described in Section 2.2.2 in Chapter 2. Blood sampling methods are described in Section 2.2.3. The baseline blood sample (4 mL) from the K2 EDTA vacutainer tubes were used.

3.2.3 Arterial stiffness measurement

An indirect and non-invasive measure of arterial stiffness was obtained by the SphygmoCor XCEL device (AtCor Medical Pvt, Ltd, Sydney, NSW, Australia). The SphygmoCor device was used to determine arterial stiffness parameters, including carotid-femoral arterial pulse wave velocity (PWV), augmentation pressure (AP) and augmentation index (AIx), and central and brachial blood pressures at the arm. The participants rested quietly in a supine position at room temperature for 15 min before measurements of arterial pulse pressure (PP) waveforms, by an inflated cuff at the brachial artery. SphygmoCor system calculates the central aortic AP by subtracting the pressure at the first systole resulting from the return of the reflected wave from the systolic pressure. AP is an indicator of arterial stiffness, as lower arterial compliance and elasticity results in an earlier return of the reflected wave, causing a disproportionate rise in systolic pressure (181). Arterial stiffness indicator AIx is a measure of the degree to which the peak of a measured pressure wave is over and above the peak of the incident pressure wave, as a result of the reflected wave. AIx is automatically calculated by the SphygmoCor for a central aortic pressure waveform as the ratio of AP to PP (388).

PWV was measured simultaneously with pressure transducers, by acquiring a carotid pulse by applanation tonometry and a femoral pulse by volumetric displacement, within a cuff around the upper thigh (femoral artery) (389, 390). Three surface distances were measured using a measurement tape (SECA) and entered into the SphygmoCor system to indicate the distance from the suprasternal notch to the top of the thigh cuff, the site of carotid tonometry to the suprasternal notch, and the site where the femoral artery could be applanated by tonometry to the top of the femoral cuff. PWV was calculated by measuring the foot-to-foot transit time of the waveform between the carotid and femoral pulse and dividing it by the measured distance. The pulse waves were captured electronically on a computer using the SphygmoCor system and accepted by the system after consistent high-quality waveforms were measured. The procedures of arterial stiffness measurements do not require any familiarization, but the procedures were described with photographs to the participants during the briefing session on a separate day before the day of the trial. PWV and AIx measurements were non-invasive and the participants rested in a supine position for 15 min, in low lighting, before the measurements to calm them down. Some participants fell asleep during the measurement, which took about 15 – 20 minutes to complete. Three to five measurements were taken for each subject and the agreements in the scores were used to calculate the PWV and AIx.

3.2.4 Biochemical analysis

To minimize batch variation, sample allocation within and between plates were random, and all samples were assayed after sample collection for both Chapter 2 and Chapter 3 were completed.

3.2.4.1 Plasma FGF21

Fasted human plasma FGF21 concentration was measured with the detailed methodology described in section 2.2.4.1 in Chapter 2. Intra- and inter-assay variation for FGF21 concentration was <15%, and human plasma samples were not diluted.

3.2.4.2 Plasma Adiponectin

Fasted human plasma adiponectin concentration was measured with the detailed methodology described in the “Methods” section 2.2.4.3. Intra- and inter-assay variation for adiponectin concentration was <15%, and plasma samples were diluted 500 fold.

3.2.4.3 Plasma oxLDL

Fasted human plasma oxLDL concentrations were measured using commercially available ELISA assay according to the manufacturer’s instructions (Mercodia oxLDL ELISA, Sweden). Briefly, the plasma samples were diluted 1:81 twice to arrive at a final dilution of 1:6561, with sample buffer diluted to a working concentration. Assay standards of 19.5, 10.3, 5.64, 2.65, 1.35 mU/L, calibrator 0 (negative control), samples and controls (25 µL) were added into each well of the 96-well microtiter plate in duplicates, followed by 100 µL of assay buffer. The microtiter plate was then incubated on a plate shaker at room temperature (800 rpm) for 2 h. The plate was then manually inverted and washed six times by pipetting 350 µL of wash buffer into each well during each wash. Thereafter, 100 µL of enzyme conjugate was added into each well and incubated at room temperature (800 rpm) for 1 h. After the incubation, the plate was washed six times before adding 200 µL of substrate solution. The plate was left on the bench for 15 min at room temperature without shaking, before adding 50 µL of stop solution. Immediately after, the plate was left on a shaker at 800 rpm for five seconds to ensure proper

mixing before measuring the treated samples and standards at an optical density of 450 nm with a microplate reader (Synergy H1, BioTek). Plasma oxLDL concentrations were determined by matching the OD samples against a cubic spline regression equation established with the standard curve as per manufacturer's instructions.

3.2.5 Statistics

Statistical methodology and sample size calculation have been previously described in Section 2.2.6. Blood-related and arterial stiffness-associated parameters were also analysed using two-way ANOVA (Age $\times$ Activity). Due to the skewed data, a logarithmic transformation was used for PWV and FGF21 concentration, while adiponectin concentration was transformed using the cube-root function. Unless otherwise stated, sex was adjusted by treating it as a categorical control variable in the ANOVA.

3.3 Results

3.3.1 Participant demographics

The demographic, physical and blood pressure profiles of the eighty participants are presented in Table 3.1. Age was equally distributed between active and sedentary participants in both younger and older groups. Sex was equally matched between the YA and YS group, while there was one more male participant in the OA than in the OS group.

3.3.1.1 Body weight, Height and Abdominal girth

There were no significant differences in bodyweight and height among different age and activity groups. Regardless of exercise habits, height was significantly lower in the older than younger adults after adjusting for sex ($p = 0.01$). However, BMI was significantly lower in the younger than the older group ($p = 0.003$) independent of activity level. Compared with

younger participants, older participants also had higher waist circumferences ($p < 0.001$) independent of exercise habits (Table 3.1). Adjusting for sex did not affect any of the other parameters described.

3.3.1.2 Blood Pressure and resting heart rate

Compared with the younger participants, older participants had 10% and 13% higher brachial and central systolic BP ($p < 0.001$) regardless of activity level. Both brachial and central diastolic BP increased by 12% and central PP increased by 15% with age (all $p < 0.001$), independent of exercise habits. Older participants also had 11.5% higher MAP than their younger counterparts ($p < 0.001$)

Brachial and central diastolic BP were both 6% lower in active than sedentary groups ($p = 0.006$), but central PP increased by 7% with habitual exercise ($p = 0.021$), regardless of age. MAP decreased by 6% in the active groups ($p = 0.003$). Resting heart rate was 15% lower in active than sedentary groups, independent of age (Activity $p < 0.001$). No significant interaction effect was found in any of the BP parameters and for resting heart rate. Adjusting for sex did not affect these results.

Table 3.1 Mean (SD) of participant demographics, exercise history, anthropometry, pulse wave analysis and resting heart rate

	Young Sedentary (YS)	Young Active (YA)	Older Sedentary (OS)	Older Active (OA)	Age	Act	Age × Act
<i>n</i>	20	20	20	20			
Sex <i>n</i> (M/F)	10/10	10/10	11/9	12/8			
Exercise intensity	Light	Mod-High	Light	Mod-High			
Exercise (min/week)	18 (27)	369 (177)	16 (28)	350 (214)	0.747	<0.001	0.790
Age (years)	28 (5)	28 (5)	56 (7)	57 (9)	<0.001	0.668	0.668
Weight (kg)	59.3 (7.2)	60.4 (13.8)	64.3(13.5)	62.9 (11.8)	0.150	0.937	0.619
Height (cm)	169.6 (8.2)	166.8 (10.1)	163.9 (8.7)	167.5 (8.6)	0.208	0.852	0.116
BMI (kg/m ²)	20.6 (2.2)	21.5 (2.2)	23.8 (4.0)	22.2 (2.4)	0.003	0.563	0.053
WC (cm)	72.9 (5.3)	72.8 (7.0)	82.1 (9.2)	77.8 (8.5)	<0.001	0.207	0.216
Br BP Systolic (mmHg)	109 (8)	110 (5)	121 (11)	119 (12)	<0.001	0.891	0.577
Br BP Diastolic (mmHg)	67 (7)	64 (5)	76 (8)	70 (8)	<0.001	0.006	0.247
Br PP (mmHg)	38 (9)	39 (9)	38 (8)	42 (12)	0.500	0.229	0.469
Ar BP Systolic (mmHg)	96 (7)	95 (5)	110 (9)	106 (8)	<0.001	0.178	0.305
Ar BP Diastolic (mmHg)	67 (8)	65 (5)	77 (8)	71 (8)	<0.001	0.006	0.234
Ar PP (mmHg)	29 (4)	31 (4)	33 (4)	35 (5)	<0.001	0.021	0.886
MAP (mmHg)	80 (8)	76 (5)	90 (9)	83 (7)	<0.001	0.003	0.383
Resting HR (beats/min)	65 (10)	56 (8)	66 (10)	56 (6)	0.926	<0.001	0.916

Body mass index (BMI), hip circumference (HC), waist circumference (WC), brachial (Br) and central aortic (Ar) systolic and diastolic blood pressure (BP), pulse pressure (PP), mean arterial pressure (MAP) and resting heart rate (HR), Mod = moderate.

3.3.2 Effects of habitual exercise and ageing on arterial stiffness

Compared with the younger participants, in older participants, arterial stiffness parameters including AIx, which was consistently higher by 3.4-fold ($p < 0.001$), and PWV, which was

also 1.2-fold higher ($p < 0.001$) (Figure 3.1A, B), independent of exercise habits. Adjusting for sex, BMI and WC did not influence the age-associated effects on arterial stiffness. However, independent of age, arterial stiffness parameters were not statistically different between active and sedentary groups, as indicated by PWV ($p = 0.09$) and AIx ($p = 0.142$), even after adjusting for sex ($p = 0.065$ and $p = 0.137$, respectively) (Figure 3.1). No significant interaction between activity and age was observed for all arterial stiffness parameters.

Figure 3.1 Mean (SD) for augmentation index (AIx) (A), pulse wave velocity (PWV) (B) in older and younger age groups, between active and sedentary individuals. *** = $p < 0.001$ for Age. Black circles represent habitually active participants and white circles represent sedentary participants.

3.3.3 Exercise and ageing on oxidative stress, FGF21 and adiponectin concentrations

Compared with their younger counterparts, older adults had 25.6% higher basal plasma oxLDL concentrations than younger adults ($p < 0.001$), independent of exercise habits, suggesting a higher oxidative stress, even after adjusting for sex, BMI and WC ($p = 0.001$). Independent of age, habitual exercise was not associated with oxLDL concentrations ($p = 0.275$), even after

adjusting for sex ($p = 0.259$). There was no significant interaction effect of age and exercise for oxLDL concentration ($p = 0.583$) (Figure 3.2A).

Plasma samples from two participants were insufficient for determination of FGF21 concentration and were excluded from the analysis. The FGF21 concentration reported are from 78 participants, YS ($n = 19$), YA ($n = 19$), OS ($n = 20$), OA ($n = 20$). The circulating FGF21 concentrations were not different between older and younger groups, regardless of activity status ($p = 0.097$) and adjusting for sex, BMI and WC did not influence the results ($p = 0.397$, Figure 3.2B). However, fasted circulating concentration of FGF21 was 9% lower in active participants than their sedentary counterparts, regardless of age ($p = 0.011$), even after adjusting for sex ($p = 0.012$). The interaction effect of age and activity were also not significant for plasma FGF21 concentration ($p = 0.475$) (Figure 3.2B)

Circulating adiponectin concentration was not significantly different between active and sedentary groups ($p = 0.821$, Fig 2B) or between younger and older groups ($p = 0.642$, Fig 2B), even after adjusting for sex, BMI and WC ($p = 0.399$). No significant interaction effect was observed for adiponectin concentration ($p = 0.418$) (Figure 3.2C).

Figure 3.2 Mean (SD) for oxidized low-density lipoprotein (oxLDL) (A), fibroblast growth factor 21 (FGF21) (B), adiponectin (C) in older and younger age groups, between active and sedentary individuals. *** = $p < 0.001$ for Age. † = $p < 0.05$ for physical activity. Grey bars represent habitually active participants and white bars represent sedentary participants.

3.3.4 Associations between FGF21, adiponectin, oxLDL and arterial stiffness

When analysed as an entire cohort, the correlations between plasma FGF21 concentration and AIx and PWV were not statistically significant, regardless of exercise habits and age (Table 3.2). However, adjusting for sex in a partial correlation analysis between FGF21 concentration

and AIx ($r = 0.254, p = 0.027$), and PWV ($r = 0.232, p = 0.044$) were statistically significant. In contrast, higher plasma adiponectin concentration was negatively associated only with PWV ($r = -0.384, p < 0.001$). Plasma oxLDL concentration was positively associated with both AIx ($r = 0.227, p = 0.043$) and PWV ($r = 0.367, p = 0.001$) (Table 3.2). There were no significant associations between plasma FGF21, adiponectin or oxLDL concentrations (all $p > 0.05$, data not shown) and adjusting for sex did not affect the results presented.

For subgroup analysis, plasma adiponectin concentration was negatively associated with PWV within the younger group ($r = -0.359, p = 0.025$), regardless of exercise habits. However, there was a stronger association between adiponectin and PWV in the older group ($r = -0.509, p = 0.001$) (Table 3.2), regardless of exercise habits. Plasma oxLDL concentration was also positively associated with plasma FGF21 concentration in the older ($r = 0.345, p = 0.029$) but not the younger group ($r = -0.132, p = 0.431$), regardless of activity status, even after adjusting for sex (Figure 3.3).

In subgroup analysis, plasma FGF21 concentration was positively associated with AIx in sedentary ($r = 0.369, p = 0.021$), but not in active participants ($r = 0.006, p = 0.970$), regardless of age (Figure 3.4, Table 3.2). There was also a negative association between plasma adiponectin concentration and PWV in the sedentary group, independent of age ($r = -0.511, p = 0.001$, Figure 3.4). In contrast, in active individuals, a positive association between plasma oxLDL concentration and PWV was found, regardless of age ($r = 0.452, p = 0.003$) (Table 3.2, Figure 3.4). Adjusting for sex in a partial correlation analysis did not affect these subgroup associations in the sedentary and active groups.

Table 3.2. Pearson's correlations (r) between plasma FGF21, adiponectin, oxLDL concentrations and arterial stiffness parameters

		AIx	PWV
Total	FGF21	0.222 (0.051)	0.220 (0.054)
	Adiponectin	0.004 (0.969)	-0.384 (<0.001)
	oxLDL	0.227 (0.043)	0.367 (0.001)
Younger	FGF21	0.093 (0.581)	-0.081 (0.634)
	Adiponectin	0.008 (0.963)	-0.359 (0.025)
	oxLDL	-0.214 (0.185)	0.157 (0.339)
Older	FGF21	0.197 (0.223)	0.262 (0.103)
	Adiponectin	0.097 (0.551)	-0.509 (0.001)
	oxLDL	0.075 (0.647)	0.174 (0.283)
Active	FGF21	0.006 (0.970)	0.083 (0.614)
	Adiponectin	0.062 (0.705)	-0.198 (0.220)
	oxLDL	0.248 (0.123)	0.452 (0.003)
Sedentary	FGF21	0.369 (0.021)	0.272 (0.099)
	Adiponectin	-0.031 (0.847)	-0.511 (0.001)
	oxLDL	0.178 (0.272)	0.259 (0.111)

Values are unadjusted Pearson's r on top and p values in brackets below. Values in bold interface represent statistically significant correlations.

Figure 3.3 Summary figure of effects of ageing on arterial stiffness. Blue directional arrows represent effects observed in older participants compared to younger participants. Solid black arrows represent known stimulatory effect whilst dotted arrows represent known inhibitory effects base on the literature. Green bi-directional arrows represent positive correlation and red bi-directional arrow represent a negative correlation, observed in older group only.

Figure 3.4 Summary figure of effects of exercise on arterial stiffness. Blue directional arrows represent effects observed in active participants compared to sedentary participants. Solid black arrows represent known stimulatory effect whilst dotted arrows represent known inhibitory effects base on the literature. Green bi-directional arrows represent positive correlation and red bi-directional arrow represent a negative correlation, observed in either sedentary (yellow) or active (purple) groups only.

3.4 Discussion

The present study is the first to investigate FGF21, adiponectin, oxidative stress and arterial stiffness parameters with different self-reported exercise habits and age groups in healthy individuals. Arterial stiffness was determined indirectly through measurements of PWV and AIx in this study. PWV and AIx are widely used surrogate markers of arterial stiffness and are useful, simple and non-invasive tools for diagnosis and risk stratification of cardiovascular diseases (391, 392). Compared with younger individuals, older individuals had higher circulating plasma oxLDL concentrations and arterial stiffness, regardless of exercise habits. This is consistent with previous findings that ageing independently increases oxidative stress by 1.2–3-fold and arterial stiffness parameters by ~2-fold (173, 393-396). In this present study, oxLDL concentration was positively related to PWV and AIx, regardless of age and activity status, implying that increased oxidative stress is associated with arterial stiffening, even in healthy individuals. Consistent with my results, oxidative stress was independently and positively associated to arterial stiffness, in adolescents, middle-aged and older adults (397-400) and in both healthy and diseased populations (401-403).

The mechanism through which oxidative stress contribute to arterial stiffness include changes in arterial structure and function. An increase in oxLDL activity leads to inhibition of eNOS phosphorylation, decreased endothelial NO synthesis and bioavailability, which contributes to impairment of endothelial-dependent vasodilation and function, in turn increasing arterial stiffness (394, 404, 405). During ageing in healthy individuals, increasing oxLDL concentration was inversely related to forearm blood flow during reactive hyperaemia response, implying that oxidative stress influenced vascular endothelial function and may contribute to arterial stiffness (395, 396). Another possible mechanism is structural change through vascular calcification, which involves increased collagen synthesis in arterial smooth muscle cells and

human coronary artery endothelial cells (406, 407). In animal models, oxLDL infusion *in vivo* increased structural intima thickness by 7-fold due to an inflammatory-fibroproliferative response (408). These results collectively imply that in healthy individuals, the age-associated increase in oxidative stress increases arterial stiffness by influencing vascular structure and function.

However, plasma FGF21 concentrations were not different among the age groups in my study, independent of exercise habits. Resting plasma FGF21 concentration was not associated with plasma oxLDL concentration in this cohort of healthy individuals. This study findings imply that age-associated increase in oxidative stress was unlikely associated with FGF21. In contrast, earlier findings suggested that FGF21 concentration increased with age in healthy individuals (aged 5–80 years) (54). Earlier evidences also suggested that FGF21 was involved in regulation of oxidative stress (47, 322, 409). Increased intracellular stress signalling due to oxLDL administration resulted in increased FGF21 expression in animal cardiac endothelial cells and hepatocytes (47, 322). Administration of FGF21 also protected against oxidative stress in human endothelial cells (409), supporting the positive association of FGF21 with antioxidative properties on endothelial function and arterial stiffness, observed in male and female patients on haemodialysis (320) and patients with type 2 diabetes (321), and in obese women (410). These results supported the associated role of FGF21 in mediating age-associated increase in arterial stiffness, through reducing oxidative stress. The different findings in our study could be due to our participant inclusion criteria, which was limited to a clinically healthy (free from metabolic and cardiovascular diseases) and non-obese population. The antioxidant and cardio-protective effects of FGF21 could be more obvious only in diseased populations, which have higher oxidative stress and higher fasted circulating FGF21 concentrations (319, 411). The higher oxidative stress or FGF21 resistance in diseased states may stimulate an increase in

FGF21 concentration and its antioxidative properties, potentially because the endogenous antioxidants are unable to reduce oxidative stress. Most of the mechanistic studies involving FGF21 and oxidative stress are in animal or *in vitro* models, which are difficult to translate to healthy participants in this study. Contrary to the predicted anti-oxidative properties of FGF21 in attenuating arterial stiffness, this study showed no age-associated changes in FGF21 concentration in healthy young and older adults, suggesting that FGF21 is unlikely involved in age-associated increase in oxidative stress and arterial stiffness in this population.

In this study, when analysed as an entire cohort, concentration of the FGF21-downstream-effector adiponectin (48, 297, 329), was negatively correlated to PWV, suggesting that adiponectin could be involved in arterial stiffening even in healthy individuals. Adiponectin could improve arterial stiffness through anti-oxidative properties (387, 412, 413). In obese mice, adiponectin reduced systemic oxidative stress and normalized endothelial cell function through increased NO production, which could in turn affect arterial function and reduce arterial stiffness (179, 414, 415). In humans, an inverse relationship was also reported between plasma adiponectin concentration and PWV, supporting the potential role of adiponectin in improving arterial stiffness (381, 416). The negative association between plasma adiponectin concentration and PWV in this study was more obvious in older than the younger group. Independent of adiponectin, plasma FGF21 concentration was also positively associated to oxLDL concentration only in the older group. In healthy older but not in younger adults, the unique FGF21- and adiponectin-associated mechanisms on oxidative stress and arterial stiffness respectively, suggested that different mechanisms may drive arterial stiffness during ageing. For example, in younger individuals, arterial stiffness increased due to an increase in magnitude of wave reflection, whereas in older individuals, arterial stiffness was a result of increased wave velocity resulting from less compliant aorta (173, 393). Therefore, adiponectin

may likely regulate age-associated arterial stiffening, independent of FGF21, through potential upstream mechanisms such as growth factors and inflammatory cytokines including interleukin 6 (417, 418).

The oxLDL concentration and PWV were not statistically different between habitually active and sedentary groups, suggesting that habitual exercise may not affect oxidative stress and arterial stiffness, independent of age. Inconsistent with my results, previous studies reported an inverse association between habitual exercise participation (especially vigorous intensity exercise) and arterial stiffness in healthy young adults (205, 419). In healthy middle-aged and older adults, being active and engaging in mild intensity exercise resulted in 7% reduction in arterial stiffness (420, 421). These results suggest that habitual exercise improves arterial stiffness regardless of age. The exercise-induced improvement in arterial stiffness could be mediated by a reduction in oxidative stress. For example, oxLDL was reduced with 6 weeks of exercise and dietary intervention in obese children (422). The decrease in oxLDL concentration was also inversely associated with improved endothelial function (422), suggesting that exercise could improve arterial stiffness through reduced oxidative stress and improved endothelial function. These results were also found in adult men and women (aged 18–65 years), as 4-week of moderate intensity exercise intervention decreased oxLDL concentration by 14% (423). The differences between study findings may be due to the use of self-reported exercise habits to classify exercise behaviour in this study, which might not be comparable with data collected from more objective measures, such as with the use of accelerometers in other studies to monitor physical activity (420, 421). The cross-sectional study design of the present study could have also contributed to the disparity in results with earlier intervention studies (422, 423). Exercise likely attenuates arterial stiffness through the reduction of visceral adiposity-mediated oxidative stress (424-426), which were also similar between active and

sedentary groups in this study. Hence, within a healthy population, habitual exercise unlikely influences oxidative stress and arterial stiffness.

Despite no difference in arterial stiffness, basal plasma FGF21 but not plasma adiponectin concentration, was significantly higher in sedentary than that in active individuals, regardless of age. Both FGF21 and adiponectin concentrations were also independently associated with arterial stiffness in sedentary individuals, but these relationships were not observed in active individuals. In sedentary individuals, plasma FGF21 concentration was positively associated with AIX, but plasma adiponectin concentration was inversely associated with PWV. These preliminary findings suggested that FGF21 and adiponectin, may independently influence arterial stiffness in sedentary individuals. In contrast, a previous study showed that serum FGF21 concentration was not associated with AIX in healthy controls and patients with type 2 diabetes (427), although a positive association between serum FGF21 concentration and PWV was found in both obese and diabetic individuals (321, 410). The differences in results could be due to different measures of arterial stiffness, as PWV measures static properties such as distensibility, whereas AIX measures dynamic properties including the endothelial function and peripheral resistance (227-229). Furthermore, both single-bout and chronic (1–6 weeks) exercise interventions independently decreased circulating FGF21 concentrations and increased adiponectin concentrations (290, 335, 336, 352, 428, 429), suggesting that exercise status could affect the associations between arterial stiffness parameters with both FGF21 and adiponectin concentrations. It is possible that in healthy sedentary individuals, FGF21 and adiponectin independently affect arterial stiffness.

A key limitation of this study was the cross-sectional design. Although associations can be drawn from the study results, the cross-sectional design does not conclusively prove any

causality in inferring that an increase in oxLDL and FGF21 concentrations or a decrease in adiponectin concentration was involved in the state of arterial stiffness. Given that plasma oxLDL concentration was measured as a marker of oxidative stress, the exact mechanisms driving the increase in oxidative stress on arterial stiffness cannot be determined from this study. Future studies are required to better elucidate the role of FGF21 and adiponectin on oxidative stress and arterial stiffness. Arterial stiffness was determined indirectly through measurements such as PWV in this study. Although PWV is the most widely used measure of arterial stiffness and a useful, simple and non-invasive tool for diagnosis and risk stratification of cardiovascular diseases, future studies should also consider the effects of FGF21 and adiponectin on other important measures of vascular endothelial function such as flow mediated dilation.

In summary, this study demonstrated that oxidative stress may drive age-associated arterial stiffness, unlikely through FGF21 and adiponectin. The independent associations between FGF21 and adiponectin with oxidative stress and arterial stiffness, respectively, were found in older adults only (Figure 3.3). Self-reported exercise habits unlikely attenuate oxidative stress or arterial stiffness in healthy individuals, although in the sedentary group, FGF21 and adiponectin were independently associated with arterial stiffness (Figure 3.4). This study proposed a novel FGF21 and adiponectin link to oxidative stress and arterial stiffness, evident in older and sedentary individuals.

Chapter 4: The FGF21-associated effects on age-related decline in BMD

4.1 Introduction

Age-related bone loss occurs after the age of 50 years. Low bone mass and declining bone mineral density (BMD) increase the risk of fracture and bone fragility, especially in older people (61, 63). Globally, ~200 million people are affected by the loss of BMD, and this equates to ~9 million fractures annually, commonly at the lumbar spine and hip (11, 63). During ageing, the balance of bone remodelling and skeletal regeneration shifts towards favouring greater bone resorption over bone formation (6). The bone remodelling process involves removal of “old” bone mineral during bone resorption by osteoclasts and formation of new bones through mineralization of the bone matrix by osteoblasts. This process is essential for repairing bone microdamage and maintaining bone mineral homeostasis and bone strength (66, 67).

The heritability of BMD and bone mass is suggested to be 50–85%, and the genetic effects are evident across all ages and skeletal sites, and in both sexes (109, 110). For example, twin studies have demonstrated that the heritability of hip and wrist fracture is 47–54% and that 40% of the variance in bone loss at the forearm and lumbar spine is also genetically determined (111, 115, 116). A Mexican-American family-based cohort study (>45 years) reported a 42–44% heritable component to a 5-year change in BMD at the spine and hip (113). Because bone mass and BMD are influenced by the balance between bone resorption and formation (61, 117), similarity of BMD between family members may reflect the heritability of bone remodelling and turnover markers (118). For example, a 74% heritability component for the bone formation marker bone-specific alkaline phosphatase (BAP) was reported in female twin pairs (119).

Bone resorption markers in postmenopausal twins, aged between 45 and 69 years, also have a 39–55% heritability component (120). Taken together, these studies suggest that in addition to environmental factors, heritability can affect BMD. It is therefore important to consider the genetic influence on BMD when evaluating the mechanisms driving the age-associated decline in BMD.

Fibroblast growth factor 21 (FGF21), an endocrine hormone produced by the liver, is involved in glucose homeostasis and lipid metabolism (52). Pharmacological administration of FGF21 represents a potential new treatment for type 2 diabetes and obesity (52). However, pharmacological treatment with FGF21 or FGF21 overexpression in mice result in decreased bone mass and severe bone loss, possibly by inhibiting osteoblast differentiation (51). FGF21-knockout mice exhibit decreased bone resorption and increased bone mass and bone formation, possibly because of reduced availability of receptor activator of nuclear factor kappa-B ligand (RANKL) (51). RANKL is a key factor in osteoclast differentiation and function (85). RANKL is produced by dying osteocytes and osteocyte apoptosis increases with advancing age, suggesting that RANKL concentrations increase during ageing (85, 86). In transgenic mice with FGF21 overexpression, RANKL-mediated osteoclast differentiation also increases, as evidenced by greater expression of the mRNA for osteoclast marker tartrate-resistant acid phosphatase 5b (TRAP5b) (350).

Administration of an FGF21 analogue in humans has adverse effects on bone turnover, such as increased concentrations of bone resorption markers and decreased concentrations of bone formation markers (52, 53). An inverse association between fasting plasma and serum FGF21 concentration and BMD has also been found in humans (346, 347). However, administration of an FGF21 analogue in non-human primates had no effects on bone mineral content (BMC)

and BMD, despite a two-fold increase in bone resorption marker C-telopeptide of type 1 collagen (CTX) concentration (52, 349). It is plausible that FGF21 reduces the trabecular BMD but not the cortical BMD and thickness, as shown in rats (348). These results support the idea that increased FGF21 expression can cause or contribute to skeletal fragility (51, 52).

Liver-derived protein insulin-like growth factor binding protein 1 (IGFBP1) is an essential mediator of FGF21-induced bone resorption and bone loss (350). Lower expression of liver IGFBP1 mRNA was observed in FGF21-knockout mice, whereas IGFBP1 mRNA was increased in FGF21-overexpression mice (350). Similarly, in the same study, FGF21-induced bone resorption and bone loss was completely abolished in IGFBP1-knockout mice. Administration of recombinant IGFBP1 into wild-type mice increased bone resorption marker CTX by 110% but did not affect osteoblast differentiation, whereas loss-of-function of IGFBP1 through administration of IGFBP1 blocking antibody preserved bone mass (350).

Higher serum IGFBP1 level was negatively associated with BMD in 3057 elderly European men aged 40 to 79 years (430). Compared with twins with normal weight, growth-restricted twins have higher IGFBP1 concentration coupled with lower concentration of bone formation marker pro-peptide of type 1 collagen (351). These studies suggest that IGFBP1 may mediate the FGF21-induced bone loss observed in animal models. IGFBP1 likely promotes bone resorption by binding to the integrin β 1 receptor on osteoclast precursors, which increases RANKL-stimulated osteoclastogenesis (350). RANKL binds to the RANK receptor on osteoclast precursors and activates osteoclast differentiation, thereby increasing bone resorption (85). However, IGFBP1 alone is insufficient for triggering the entire osteoclast differentiation program, which also requires the presence of RANKL (350).

To summarise, the current experimental evidence suggests that the effects of FGF21 on bone resorption are likely to be mediated through hepatic IGFBP1, which stimulates RANKL-mediated differentiation. However, the associations of FGF21–IGFBP1–RANKL regulatory axis with bone resorption and turnover has not been investigated in humans. Given that ageing is an independent risk factor for low bone mass, the FGF21–IGFBP1–RANKL pathway may be related to age-associated changes in bone turnover and decline in BMD. Therefore, the main aim of the study was to compare the associations of the FGF21–IGFBP1–RANKL regulatory axis with bone turnover and BMD between younger and older genetically linked family pairs. I hypothesised that FGF21, IGFBP1 and RANKL concentrations would increase with age. I also hypothesised that the FGF21–IGFBP1–RANKL axis would result in greater bone turnover, through increased bone formation and resorption, resulting in decreased BMD during ageing.

4.2 Methods

4.2.1 Research participants

Twenty genetically linked family pairs (parent and child) from Singapore were recruited. Younger participants were 22–39 years old and older participants were 60–71 years old. All participants were non-smokers and had no history of heart or musculoskeletal diseases. Participants with hypertension and hypercholesterolemia controlled their condition with medications. None of the female participants were taking oral contraceptives or hormone-replacement therapy. The participants were recruited through posters, social media and community outreach events. All participants were briefed on the nature and risks involved in the study and their rights to withdraw their participation without obligation before giving their written informed consent to participate in the study. The procedures in the study were approved by the institutional review board of Nanyang Technological University, Singapore (IRB-2017-12-010).

4.2.2 Study design and procedures

The study design and anthropometry measurements are described in Section 2.2.2 in Chapter 2. On the trial day, the participants arrived at the laboratory between 0900 h and 1000 h after having consumed a light meal >2 h before the trial schedule. Blood sampling methods are described in Section 2.2.3. A blood sample (9 mL) was taken by venepuncture from a forearm vein into chilled serum vacutainer tubes by a qualified phlebotomist. The global physical activity questionnaire is an internationally validated instrument for estimating weekly physical activity levels. The instrument collected information on the frequency and duration of moderate and vigorous intensity physical activity on a typical week, during work, transport (to and from places), and recreational time. Total metabolic equivalent task (MET) min/week was calculated by assigning a value of 4 METs for moderate-intensity activities and 8 METs for vigorous-intensity activities, and multiplying it by the total duration spent per week on physical activity (431).

4.2.3 Dual-energy X-ray absorptiometry (DEXA) measurements of BMD and body composition

BMD was measured using the Horizon DEXA machine (Hologic Inc., Bedford, MA, USA) following standard protocols. Each participant was scanned by the same radiographer. The participants were instructed to assume a supine position with arms by their sides and palms in a neutral position for the whole-body scan. The body composition measurement method was calibrated by a direct comparison of the participant's dual-energy attenuation values with the values produced by a phantom comprising materials with equivalent amounts of fat and lean tissue, such as plastics, that have x-ray attenuation characteristics similar to those of soft tissues in humans. The fat and lean masses of each soft tissue pixel in the participant's image was calculated using a direct pixel-by-pixel comparison with measurements obtained from the

tissue-equivalent plastic. DEXA was also used to measure BMD at the hip (total hip, femoral neck) and lumbar spine (anteroposterior L₁–L₄). After all scans were completed, the Hologic APEX software, version 5.6 (Hologic Inc., Bedford, MA, USA) was used to determine total fat, visceral fat, lean tissue, bone BMC and BMD.

4.2.4 Bioassays

4.2.4.1 Serum FGF21 concentration

Human serum FGF21 concentration was measured with the detailed methodology described in section 2.2.4.1 in Chapter 2. Intra-assay variation for FGF21 concentration was <10%, and human serum samples were not diluted.

4.2.4.2 Serum IGFBP1 concentration

Human serum IGFBP1 concentration was measured using commercially available enzyme immunoassay optimized for human serum (11-IGFHU-E01; Alpco, Salem, NH, USA). Before the start of the assay, a working concentration of conjugate solution was made by diluting the concentrate conjugate by a 1:100 ratio with the assay buffer. Standards, controls and undiluted samples from fresh aliquots were then added to each well in duplicates (25 µL each well). Assay buffer (100 µL) was then added into each well, before incubating on a plate shaker at 200 rpm for 30 min at room temperature. After incubation, the solution in the wells of the plate was manually decanted, 300 µL of wash buffer, diluted to a working 1:10 concentration with deionized water, was added to each well, and the process was repeated thrice. One hundred microlitres of the diluted conjugate was then added to each well using a multichannel pipette and the plate was incubated on a plate shaker at 200 rpm for 30 min at room temperature. The plate was subsequently washed three times as previously described, and 100 µL of substrate solution was added to each well. The plate was then incubated on the shaker for 15 min at room

temperature at 200 rpm. Stop solution (50 μ L) was added to each well, and the plate was read with a microplate reader at 450 nm (Synergy H1, BioTek, Winooski, Vermont, USA). Serum IGFBP1 concentration was determined by interpolating the optical density (OD) of the samples against a plotted linear log–log curve for the OD of standards on the y-axis and the concentrations of standards on the x-axis. The concentrations for both the high and low controls were within the acceptable range provided by the manufacturer. Intra-assay variation for IGFBP1 concentration was <5%.

4.2.4.3 Serum total RANKL concentration

Human serum total RANKL concentration was measured using a commercial sandwich enzyme immunoassay according to manufacturer's instructions (RD193004200R; BioVendor, Brno, Czech Republic). Briefly, the lyophilized master standard was reconstituted with dilution buffer (supplied) with occasional gentle shaking for 15 min before the assay to form the 32 pmol/L standard. The stock was then serially diluted to assay standards of 16, 8, 4, 2, and 1 pmol/L. Serum samples were then diluted 100-fold with a 5 μ L sample and 495 μ L of dilution buffer and mixed thoroughly with a vortex. Following this step, 100 μ L of the standards, dilution buffer (blank) and samples were added in duplicate, and the plate was incubated on the plate shaker at 300 rpm for 18 h at 4°C. Biotin-labelled antibody solution was then diluted 100-fold with biotin–antibody diluent and incubated for 18 h at room temperature. Wash solution was diluted 10-fold with a 100 mL of wash solution concentrate to 900 mL of deionized water. The plate was incubated overnight, after which the solution in the wells was manually decanted and washed five times with 350 μ L of the working concentration of wash solution added to each well using a multichannel pipette. During washing, the plate was left to stand for ~15 s after the addition of wash solution before it was manually inverted and tapped strongly against paper towels to remove all liquid from the wells. Working concentration of

biotin-labelled antibody solution were added to each well (100 μ L), and the plate was incubated on a plate shaker at 300 rpm for 1 h at room temperature. The plate was then washed five times as previously described, 100 μ L of conjugate solution was added to each well, and the plate was incubated on a plate shaker at 300 rpm for 1 h at room temperature. The plate was washed five times, and substrate solution (100 μ L) was added. The plate was sealed and covered with aluminium foil to protect it from light, incubated for 25 min at room temperature without shaking, after which 100 μ L of stop solution was added to each well and the OD was read on the microplate reader at 450 nm and 630 nm (Synergy H1, BioTek, Winooski, Vermont, USA) immediately after.

The concentration of RANKL was calculated by matching the OD of samples against a four-parameter logistic curve established using the standards, according to the manufacturer's instructions (BioVendor). The four-parameter logistic curve is a regression model that follows a sigmoidal curve. The standard curve and sample concentrations was generated by subtracting the OD readings at 630 nm from the OD readings at 450 nm. The corresponding standard and sample wells were then corrected for background by subtracting the OD values of control (blank) wells. The best fit four-parameter logistic standard curve was generated using Prism software (GraphPad, version 7.0, San Diego, CA, USA), and sample RANKL concentrations were interpolated from the sample OD readings. Intra-assay variation for RANKL concentration was <20%.

4.2.4.4 Serum TRAP5b concentration

Human serum TRAP5b concentration was measured using an immunocapture enzyme assay system following manufacturer's instructions (TRAP5b EIA MicroVue, Quidel, San Diego, CA, USA). Briefly, lyophilized standards and controls were reconstituted by adding 400 μ L of

deionized water to the vial, allowing it to dissolve for 15 min and then mixing the vial thoroughly before use. Sample diluent (100 μ L, supplied) was added to each well using a multichannel pipette, and 50 μ L of reconstituted standard, control or undiluted serum sample was added to each well in duplicate. The plate was sealed and incubated on a microplate shaker at 700 rpm for 1 h at room temperature. Wash buffer was then diluted 10-fold with 100 mL of wash buffer to 900 mL of deionized water, and the plate was manually inverted to remove the liquid in the wells. Three hundred microlitres of wash buffer was added manually to each well, the solution was decanted, and the plate was tapped on a paper towel to expel the remaining liquid. The washing step was repeated three times and 100 μ L of working substrate solution (diluted before by mixing one vial of substrate reconstitution buffer to one vial of substrate concentrate) was added to each well. The microplate was sealed, incubated at 700 rpm on a plate shaker for 30 s and then incubated in an incubator for 60 min at 37°C. Stop solution was added to each well (50 μ L) using a multichannel pipette, and the OD was read at 405 nm on a microplate reader (Synergy H1, BioTek, Winooski, Vermont, USA). The concentration of TRAP5b was determined by interpolation from a quadratic curve fit plotted using OD and the concentrations of the given standards (Prism, GraphPad, version 7.0). The concentrations of both the high and low controls were within the acceptable range provided by the manufacturer. Intra-assay variation for TRAP5b concentration was <5%.

4.2.4.5 Serum BAP concentration

Human serum BAP concentration was measured using a commercially available immunoenzymatic assay, according to manufacturer's instructions (Ostase BAP EIA, immunodiagnostic systems, Boldon Colliery, UK). Blank (0 concentration), standards (calibrators 1–5, supplied) and undiluted samples (50 μ L each) were added in duplicate to the coated microplate (supplied), and 100 μ L of conjugate solution was added to each well using

a multichannel pipette. The plate was then incubated on a microplate shaker at 700 rpm for 1 h at room temperature. Wash solution was diluted 20-fold to a working concentration by mixing 50 mL of wash solution concentrate to 950 mL of deionized water. The contents of the microplate were aspirated manually, 300 μ L of diluted wash solution was added to each well, and the plate was quickly inverted and tapped firmly on a paper towel to dry the wells. The washing process was repeated a total of three times, and 150 μ L of substrate reagent was added immediately after the final wash. The plate was then incubated on a plate shaker at 700 rpm for 15 min at room temperature. Quench reagent (100 μ L, supplied) was added to each well, and the plate was read at 405 nm and 630 nm using a microplate reader (Synergy H1, BioTek, Winooski, Vermont, USA) to determine the OD. To calculate the concentrations of BAP, the OD readings at 630 nm were subtracted from those at 450 nm, and a standard curve was generated using a point-to-point curve through the calibration points. The sample concentrations were then determined by directly interpolating the sample OD from the standard curve, without subtracting the blank (as per manufacturer's instructions). The concentrations for both the high and low controls were within the acceptable range provided by the manufacturer. Intra-assay variation for BAP concentration was <5%.

4.2.5 Statistical analysis

All statistical analyses were performed using the IBM SPSS, version 23 (IBM Corp., Armonk, NY, USA) and Prism version 7.0 (GraphPad). A sample size calculation before the study indicated that 40 participants (20 per group) was needed for the trial to have 80% statistical power to detect a two-sided hypothesis test at an α level of 0.05, with an effect size of 0.45 (54) (G*Power, version 3.1, Germany). Numerical variables are presented as mean (standard deviation, SD) in the text and figures unless otherwise stated. The participants' characteristics, including the anthropometric variables, blood pressure, body composition and BMD, were

analysed using paired t tests to identify differences between younger and older linked family pairs. Because of the skewed distribution of the data, a logarithmic transformation was performed for the serum FGF21, IGFBP1, TRAP5b and RANKL concentrations, and for BMD (total hip, femoral neck and lumbar spine) values. Similarly, paired t tests were used to identify differences in serum FGF21, IGFBP1, RANKL, TRAP5b and BAP concentrations between younger and older family pairs. Pearson's correlational analysis was used to evaluate associations between FGF21, IGFBP1, RANKL, TRAP5b and BAP concentrations and BMD at various sites (total hip, femoral neck, lumbar spine). Adjusting for sex in an ANOVA with age as fixed effects and sex as a categorical control variable, or by including sex in a partial correlation analysis, did not alter the significance for the results presented, and sex was therefore excluded from the analysis. All results in the tables and figures are presented as untransformed data. A p value < 0.05 was considered to be significant.

4.3 Results

4.3.1 Study sample and analyses

Younger adults were taller than older adults ($p < 0.001$) but body weight ($p = 0.666$) and BMI ($p = 0.07$) did not differ between age groups. WC did not differ significantly between younger and older adults ($p = 0.122$). Visceral adiposity was 2.1-fold higher in older than in younger adults ($p < 0.001$). Similarly, absolute fat mass was 29% higher and percentage body fat was 9.3% higher in older than in younger adults ($p = 0.004$ and $p < 0.001$, respectively). Compared with the younger adults, older adults had 14.6% lower lean body mass ($p = 0.04$), 9% lower total BMD ($p = 0.009$) and 19% lower total BMC ($p = 0.002$). The older adults also had 9% higher systolic blood pressure than younger adults ($p = 0.015$), but similar diastolic blood pressure ($p = 0.138$). None of the participants had osteoporosis, as indicated by the T-scores and Z-scores, at the measured sites, including the spine, hip and femoral neck (Table 4.1).

There was no difference in weekly activity status between younger and older family pairs (Table 4.1).

Table 4.1 Mean (SD) of participant demographics, anthropometry, blood pressure and BMD scores

	Younger	Older	<i>p</i> value
<i>n</i>	20	20	
Sex <i>n</i> (M/F)	9/11	4/16	
Menopause (%)	0	100	
Race (Chinese/Malay)	18/2	18/2	
Activity (MET min/wk)	1693 (1598)	1912 (1345)	0.652
Age (years)	29 (5)	63 (2)	<0.001
Weight (kg)	59.8 (11.9)	58.1 (12.8)	0.666
Height (cm)	166.5 (8.3)	156.3 (8.8)	<0.001
BMI (kg/m ²)	21.4 (2.9)	23.6 (3.6)	0.073
WC (cm)	72.6 (10.0)	78.1 (11.9)	0.122
Systolic BP (mmHg)	112 (11)	122 (16)	0.015
Diastolic BP (mmHg)	70 (8)	74 (8)	0.138
Fat mass (kg)	17.1 (5.4)	22.1 (5.3)	0.004
% Body fat	28.8 (6.9)	38.1 (5.5)	<0.001
Lean body mass (kg)	40.3 (9.5)	34.4 (9.1)	0.04
Visceral adiposity (g)	336.9 (140.9)	704.3 (271.2)	<0.001
Total BMD (g/cm ²)	1.12 (0.1)	1.02 (0.13)	0.009
Total BMC (g)	2185 (441)	1780 (395)	0.002
T-scores and Z-scores			
Spine T-score		−1.5 (1.1)	
Spine Z-score	−0.7 (1.1)		
Total hip T-score		−1.1 (0.7)	
Total hip Z-score	−0.2 (1.2)		
Femoral neck T-score		−1.6 (0.6)	
Femoral neck Z-score	−0.3 (1.4)		

Body mass index (BMI), waist circumference (WC), systolic and diastolic blood pressure (BP), body mineral density (BMD) and bone mineral content (BMC) for younger and older family pairs.

4.3.2 Bone mineral density

Compared with the younger adults, older adults had 10% lower BMD at the lumbar spine ($p = 0.007$), 13% lower BMD at the hip ($p = 0.008$) and 18% lower BMD at the femoral neck ($p = 0.001$) (Figure 4.1).

Figure 4.1 Mean and individual bone mineral density at the lumbar spine (A), hip (B) and femoral neck (C) between genetically linked family pairs. ** $p < 0.01$

4.3.3 FGF21, IGFBP1, RANKL and bone turnover markers

Serum FGF21 concentration was three-fold higher in older than in younger participants ($p < 0.001$, Figure 4.2A). However, serum IGFBP1 concentration was similar in younger and older adults ($p = 0.961$, Figure 4.2B). Circulating total serum RANKL concentration was 44% lower in older than in younger adults ($p = 0.006$, Figure 4.2C). Compared with their younger counterparts, older adults had 36% higher serum TRAP5b concentration ($p = 0.01$, Figure 4.2D) and 31% higher serum BAP concentration ($p = 0.004$, Figure 4.2E).

Figure 4.2 Mean and individual circulating serum concentrations of FGF21 (A), IGFBP1 (B), RANKL (C), TRAP5b (D) and BAP (E) in genetically linked family pairs. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

4.3.4 Associations between the serum concentrations of FGF21, IGFBP1, RANKL and bone turnover markers and BMD at the femoral neck, hip and spine

FGF21 concentration correlated inversely with BMD at the lumbar spine ($r = -0.460, p = 0.003$) but not with BMD at the femoral neck ($r = -0.306, p = 0.055$) or at the total hip ($r = -0.275, p = 0.086$) (Table 4.2). IGFBP1 concentration was not associated with femoral neck BMD ($r = -0.163, p = 0.315$), total hip BMD ($r = -0.206, p = 0.203$) or spine BMD ($r = 0.233, p = 0.148$). However, RANKL concentration correlated positively with BMD at the total hip ($r = 0.345, p = 0.029$) and the spine ($r = 0.550, p < 0.001$) but not at the femoral neck ($r = 0.299, p = 0.061$). The concentration of the bone resorption marker TRAP5b correlated inversely with BMD at all sites, including the femoral neck ($r = -0.355, p = 0.025$), total hip ($r = -0.373, p = 0.018$) and spine ($r = -0.550, p < 0.001$). Similarly, the concentration of the bone formation marker BAP also correlated inversely with BMD at the femoral neck ($r = -0.417, p = 0.007$), total hip ($r = -0.479, p = 0.002$) and spine ($r = -0.448, p = 0.004$) (Table 4.2).

No association between IGFBP1 and FGF21 concentrations was observed ($r = -0.144, p = 0.374$) (Figure 4.3A). However, an inverse association between RANKL and TRAP5b concentrations was observed ($r = -0.494, p = 0.001$) (Figure 4.3B). The concentrations of the bone resorption marker TRAP5b and bone formation marker BAP correlated significantly ($r = 0.480, p = 0.002$) (Figure 4.3C).

Table 4.2 Pearson's correlations between the concentrations of FGF21, IGFBP1, RANKL and bone turnover markers with BMD at the femoral neck, hip and spine

	Femoral neck BMD	Total Hip BMD	Lumbar Spine BMD
FGF21	-0.306	-0.275	-0.460**
IGFBP1	-0.163	-0.206	-0.233
RANKL	0.299	0.345*	0.550***
TRAP5b	-0.355*	-0.373*	-0.550***
BAP	-0.417**	-0.479**	-0.448**

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 4.3 Pearson's correlations between FGF21 and IGFBP1 concentrations (A), RANKL and TRAP5b concentrations (B), and TRAP5b and BAP concentrations (C) in all participants (young and old).

Figure 4.4 Summary figure showing the associations of FGF21–IGFBP1–RANKL axis with age-related bone turnover and decline in BMD. Green arrows represent higher concentrations, red arrows represent lower concentrations and grey arrows represent no change in concentrations in older than in younger family pairs.

4.4 Discussion

The present study is the first to investigate the involvement of the FGF21–IGFBP1–RANKL axis in age-related bone turnover changes and BMD in humans. This study showed that ageing is associated with a loss of BMD and an increase in FGF21 concentration, albeit these are unlikely to be associated with IGFBP1. This study also showed an age-associated decrease in total circulating RANKL concentration coupled with an increase in the concentrations of bone resorption marker TRAP5b and bone formation marker BAP.

FGF21 is a potent regulator of skeletal homeostasis. FGF21 overexpression decreases bone mass, whereas loss of FGF21 function increases bone mass in mice (51). In animal models, the FGF21-induced pro-osteoclastogenic effects are mediated through the liver hormone IGFBP1 (350). Contrary to the predicted age-associated increase in both FGF21 and IGFBP1 concentrations along with decreased BMD in animals, this study showed no difference between older and younger adults in circulating IGFBP1 concentrations despite higher FGF21 concentrations and lower spine, hip and femoral neck BMD in the older adults. An inverse association between FGF21 concentration and lumbar spine BMD was found, regardless of sex and age, despite no association between IGFBP1 concentration with BMD and FGF21 concentrations. These results suggest that FGF21 is associated with the age-related decrease in BMD in healthy adults but that IGFBP1 is unlikely to be involved. Other investigators also report decreases in BMD at the lumbar spine, femoral neck and hip during ageing in both men and women (432, 433), with BMD inversely associated with FGF21 concentration in healthy elderly participants aged ~60 years, regardless of sex (346, 434). Experimental studies involving FGF21 administration, in both animals and humans, also resulted in lower trabecular bone volume and bone thickness, lower BMD and reduced bone formation (52, 348). Collectively, the age-associated decline in BMD is plausibly associated with the increase in

FGF21 concentration. Although the FGF21-associated decline in BMD is consistently observed in animals and humans, the evidence on the actions of the FGF21–IGFBP1 bone resorption axis are based primarily on *in vivo* animal models (350, 435).

In haemodialysis patients, circulating post-prandial FGF21 concentrations was positively associated with IGFBP1 concentration (436). IGFBP1 independently stimulates osteoclast differentiation and was positively associated with increased fracture risk in overweight elderly women (~73 years) (350, 437), which suggests the role of FGF21–IGFBP1 axis on age-associated bone loss in humans (350, 437). Serum IGFBP1 concentration was also negatively associated with bone formation and BMD at the hip and positively associated with bone loss in diabetic patients and growth-restricted twins (351, 438). Contrary to this study, these earlier results suggest that FGF21-associated bone loss is likely mediated through IGFBP1. There are insufficient studies investigating the FGF21–IGFBP1 axis on bone loss during ageing in humans, especially in a healthy population, as most of the current evidences are based on diseased populations. The different results between studies also suggest that there are other factors that influence IGFBP1 concentration during ageing besides FGF21, such as insulin-like growth factor 1 (IGF1), which concentrations decreases with age (439, 440). IGF1 binds to IGFBP1 in the circulation and functions to increase BMD, evidenced by a 10% decrease in BMD in liver-specific IGF1 knockout mice, which was restored with 4-weeks of IGF1 treatment (441). FGF21 could decrease IGF1 expression through inhibition of growth hormone, independent of IGFBP1, resulting in age-associated decline in BMD and bone mass (442). A previous study also found similar serum IGFBP1 concentrations between osteoporotic postmenopausal women and normal postmenopausal or premenopausal women, suggesting that IGFBP1 might not affect BMD (443). The finding in this current study supports that in

both healthy males and females, FGF21 may be related to the age-associated decline in BMD but IGFBP1 is not directly involved.

IGFBP1 independently binds to integrin receptors on osteoclast and facilitates *in vitro* osteoclast precursor differentiation (277, 444, 445). However, the ability of IGFBP1 to induce osteoclast differentiation requires the presence of RANKL (350). The lower circulating serum RANKL concentration in the older participants in my study contradicts the suggested function of RANKL in osteoclast formation and activation and in increasing bone resorption (85). For example, cultured cells from adult and old mice had 2.1–4.4-fold higher RANKL mRNA expression and their whole bone volume 20–52% lower than in young mice (446). However, my results agree with reports on the decline in circulating RANKL concentrations by ~13% every 5 years in healthy elderly subjects aged 81–90 years (447, 448). The mechanisms underlying the age-associated decrease in RANKL concentration in the present study is unclear and may represent the differences of RANKL between humans and animals, in mediating bone resorption with age. The current results contradict my hypothesis on the FGF21–IGFBP1–RANKL bone loss axis that RANKL concentrations increase during ageing, implying that other factors could influence RANKL concentrations and age-associated increase in bone resorption and bone loss.

The lower RANKL concentration in older participants in my results may be mediated by osteoprotegerin (OPG), which functions as a decoy receptor for RANKL by competing with RANK for RANKL binding on osteoclast precursor and inhibiting osteoclast activation (449). It is plausible that the increase in bone resorption and the corresponding decrease in BMD with ageing are the result of an increase in the RANKL/OPG ratio (450, 451), which was not quantified in the present study. In this study, ageing may be associated with a greater decrease

in OPG relative to RANKL concentrations, which would increase the RANKL/OPG ratio and stimulate osteoclast differentiation and bone resorption (450, 451). This study measured total circulating RANKL concentrations without discriminating between bound RANKL and free RANKL. It is possible that the lower total circulating RANKL concentration observed during ageing reflects less OPG bound to RANKL or greater RANKL sensitivity through increased expression of the osteoclast precursor receptor RANK. If so, the increased binding of RANKL to RANK during ageing would increase osteoclast differentiation and bone resorption. For example, *in vitro* OPG expression was 40% lower and the RANKL/OPG ratio was higher in aged mice than in young mice (452). In that study, exogenous RANKL administration increased osteoclast formation in cells. The magnitude of increase of osteoclast formation was also larger in old than in young mice, possibly because of the two-fold increase in RANK receptor expression during ageing (452).

In the present study, the age-associated decrease in circulating RANKL concentration was associated with an increase in TRAP5b concentration. Since TRAP5b is an osteoclast and bone resorption marker, my results suggest an overall increase in osteoclastic activity with age. This finding implies that despite the lower total RANKL concentration in older than younger family pairs, osteoclast differentiation may increase during ageing through increased binding of RANKL to RANK receptor on osteoclast precursors. However, there was no difference in IGFBP1 concentrations between the young and older adults. Therefore, this study supports that in healthy individuals, RANKL, but not IGFBP1, is a downstream effector of the FGF21-associated decline in BMD during ageing, likely through increased osteoclast differentiation and bone resorption.

In my study, TRAP5b and BAP concentrations were higher in older than in younger adults, which suggests an increase in both bone resorption (osteoclastic activity) and bone formation (osteoblastic activity) with ageing. Both TRAP5b and BAP concentrations were also inversely associated with BMD at the hip, femoral neck and spine and TRAP5b concentration was positively associated with and BAP concentrations. Taken together, these results suggest that FGF21-associated decline in BMD during ageing may be due to a negative balance in bone turnover despite the increase in overall bone remodelling. My findings are consistent with the increase in both BAP and TRAP5b concentrations, despite a decrease in BMD, in elderly men and women after hip replacement (76), implying that BMD in older people is determined by the balance in bone resorption and formation activity. The age-related bone loss was associated with increased bone resorption and osteoclastic activity, evidenced by an increased TRAP5b concentration in older than in younger adults (453). Therefore, the FGF21–RANKL axis possibly contributes to increased bone resorption and formation, resulting in the age-associated decrease in BMD.

Compared with elderly men with lower concentrations of bone formation markers, elderly men with higher concentrations of bone formation markers had lower areal and volumetric BMD (454). The age-associated increase in bone formation markers could reflect a response to bone loss and bone remodelling and an increase in BAP concentration and bone formation could be a compensatory response to the FGF21–RANKL-associated increase in bone resorption and decline in BMD with ageing. During fracture healing, BAP concentration was higher in people with low BMD compared with those with normal BMD, which suggests that people with low BMD require greater bone formation during bone repair (455). Both bone formation (as indicated by BAP concentration) and resorption (as indicated by TRAP5b concentration) are accelerated during periods of growth spurt (456), suggesting that bone formation and resorption

are both critical in remodelling, and plausibly influenced by FGF21-associated bone loss, especially during ageing. Therefore, although both bone formation and resorption activities increased with age, the balance of bone mineral turnover is shifted negatively to favour greater bone resorption than bone formation, resulting in declining BMD (6). The FGF21–RANKL-associated decrease in BMD and increase in osteoclastic activity implies increased bone resorption during healthy ageing. The increased osteoblastic activity and bone formation appears to be a necessary compensatory attempt to balance the remodelling process with healthy ageing.

The positive association between FGF21 concentration and age in the present study contradicted the results in Chapter 3, which found no effects of ageing on FGF21 concentration. The difference in results could be due mainly to the different recruitment criteria. Participants in Chapter 3 were free from disease, medication and medical treatment, and their health status was ascertained with medical screening, but the participants in this study included individuals undergoing treatments for hypertension and hypercholesterolemia. Participants in Chapter 3 were also not related family pairs, unlike this study, which recruited genetically-linked' family (parent and child) pairs. The condition of FGF21 measurement could have also affected the relationship between FGF21 concentration and ageing. For example, the FGF21 concentration in Chapter 3 was measured under fasted conditions, but FGF21 concentration in this study was measured >2 h after a light meal. Future longitudinal studies would be required to better elucidate the effects of ageing on FGF21 concentrations.

A key limitation of the present study is the lack of data on sex steroids, which can influence age-associated bone loss, especially in post-menopausal women with reduced oestrogen levels. Despite the inclusion of genetically linked family pairs in my study, sex was not matched

within each family pair, and men and women were not equally represented within the younger and older groups. However, statistical adjustment for sex did not influence the observed effects of ageing on the biomarkers measured in the current study. This study was explorative in nature and future studies involving a larger and sex-matched cohort would help to extend the investigation and shed more light on the FGF21-associated mechanisms involved in bone turnover. Furthermore, although the family pair design in this study minimises genetic effects, not all the family pairs who participated in the study lived together, suggesting that environmental factors such as lifestyle and dietary intake may differ between them and could have influenced the results of the study. In addition, the findings of the present study are based on this cohort of healthy people with no musculoskeletal conditions, which may not be generalised to other populations or ethnicities, as the present cohort consists of 90% Chinese. Given the cross-sectional study design, the differences in FGF21, IGFBP1, RANKL, BAP and TRAP5b concentrations between age groups are associative only and cannot be used to conclude causality in inferring that FGF21 is involved in reducing BMD. Correlations drawn from this study are also explorative and associative due to the rather small sample size. Future longitudinal studies and experimental models with a larger sample size, which account for lifestyle factors, are required to elucidate better the FGF21-associated mechanisms that drive the age-associated decline in BMD in humans. Notably, the bone turnover markers measured in this study were not influenced by feeding status (457-459) and all participants were instructed to consume specific food types at ~2 h before arrival at the laboratory. The participants also arrived at the laboratory at a specific time in the morning, which minimized the effects of diurnal variation on the biomarkers measured. The conclusions on FGF21 on bone turnover are also specific to biomarkers measured in this study, including RANKL, BAP and TRAP5b, which may not be generalised to other bone markers on osteoclast differentiation or turnover that were not measured in the present study.

In conclusion, the present study is the first to investigate the associations of the FGF21–IGFBP1–RANKL axis with bone turnover and the age-associated decline in BMD in humans (Figure 4.4). The findings from this study of genetically-linked parent and child pairs suggest that FGF21 may be involved in the age-associated decline in BMD, particularly at the lumbar spine. The results from this study do not support the association of FGF21 with downstream effector IGFBP1 and suggest that IGFBP1 is unlikely to be involved in RANKL-associated osteoclast differentiation in humans. Despite an age-associated decrease in total RANKL concentration, the concentrations of both bone formation and bone resorption markers increased with ageing; this finding supports the idea that bone remodelling is an integral process in healthy ageing. More studies are required to elucidate the potential mechanisms through which FGF21 affects BMD and bone turnover with ageing.

Chapter 5: Associations of lung microbiome with age-related changes in lung function and arterial stiffness

5.1 Introduction

Ageing is an identified risk factor for several chronic health conditions, including lung and cardiovascular disease, which exhibit disproportionate susceptibility, morbidity and mortality in older individuals (460, 461). Ageing is also associated with a decline in subclinical lung function and health (462, 463), dysfunction of vascular endothelium and arterial stiffening in the healthy population (173, 174, 464). In individuals >40 years old (42), measurements of lung function were inversely associated with arterial stiffness parameters, even after adjustment for cardiovascular risk factors, smoking history and lung disease (41, 44). Reduced pulmonary function is an independent risk factor for cardiovascular morbidity and mortality (223) and is independently associated with precursors for cardiovascular disease, including arterial stiffness, regardless of age, sex or anthropometry (40-44). Pulmonary function also predicted the development of atherosclerotic plaques and is a strong risk factor for arterial stiffness, suggesting a causal relationship between the decline in lung function and increase in arterial stiffness (40, 43, 224).

While evidence supports the relationship between declining lung function, arterial stiffness and subsequent onset of cardiovascular diseases (223), the mechanisms underlying these associations are not known presently. A possible mechanism for this relationship is inflammation, as arterial stiffness is markedly increased in patients with chronic obstructive pulmonary disease (COPD), which is characterised by airflow obstruction and inflammation (225, 226, 465). While systemic inflammation, weight, smoking history, hypercholesterolemia, hypertension and diabetes have all been implicated, they do not fully account for the

relationship between pulmonary function and arterial stiffness, suggesting that other factors could be involved (41, 223, 231).

The lung microbiome encompasses the collective genetic information of all microorganisms that colonize the lung and differs between healthy and diseased individuals (260). Lower microbiome diversity and the presence of specific microbial taxa from Proteobacteria phylum are associated with decreased lung function in pathogenic disease states (260, 270). In healthy individuals, the lung microbiome comprises a community of low density and transiently present microorganisms, whereas pathogenic organisms predominate in disease (39, 265, 268). While the effects of ageing on the lung microbiome remain to be established, the gut microbiome has been found to influence healthy ageing through alteration of inflammatory markers, diet and immune response (237, 249, 252). Furthermore, age-associated alterations in immune function may affect the microbiome (or vice versa), generating low-grade inflammation, which also contributes to arterial stiffness and declining lung function (466). An emerging question is whether the observed immune-pathological associations between gut microbiota and disease also hold true at other anatomical sites, such as the lung, where the microbiome may underpin age-associated changes in lung function with a consequent impact on arterial stiffness. This study explored the age-associated changes in lung microbiome and their links with arterial stiffness and lung function in younger and older family pairs. I hypothesised that ageing is associated with changes in the lung microbiome composition, which is associated with age-related changes in lung function and arterial stiffness.

5.2 Methods

5.2.1 Research participants

The participant criteria for recruitment are described in “Methods” section 4.2.1 in Chapter 4. Eight more participants who met the inclusion criteria were recruited for this study, resulting in a total of 24 genetically-linked family pairs (parent and child) included in the analysis. All participants were non-smokers, not on long-term inhaled medication, had no history of chronic respiratory diseases and resided in Singapore over the 10–12-month period preceding recruitment. The procedures in the study were approved by the institutional review board of Nanyang Technological University (IRB-2017-12-010).

5.2.2 Study design and procedures

The study design and anthropometry measurements are described in Section 2.2.2 in Chapter 2.

5.2.3 Sputum collection and processing

Participants rinsed their mouth with water, before careful instructions were given on how to produce sputum from a deep cough, using the huff cough method (467). Participants took three deep maximal breaths, before coughing as hard and as deeply as possible in a standing position. Sputum was collected by spontaneous expectorate from a deep cough (0.5–2 g) into sterile containers and were visually inspected before placing on ice immediately. Sputasol (ThermoFisher Scientific, Massachusetts, United States) was added into the sputum container in equal amounts as sample. The sputum sample was then shaken at 200 rpm at 37 °C for 15 min. RNALater solution (ThermoFisher Scientific, Massachusetts, United States) was added and the sample stored in 1 mL aliquots at –80 °C. Saliva samples were assessed in 6 participants (3 matched pairs) to allow comparative assessments between oral and lung sampling. Prior to

sputum collection, participants rinsed their mouth with water and saliva samples (5 mL) were collected into a 50 mL sterile tube by allowing saliva to accumulate before expectoration and the samples were placed on ice immediately (468). Saliva samples were aliquoted (500 µL) and RNALater solution (1 mL) added and samples stored at –80 °C.

5.2.4 Spirometry

Lung function measurement was conducted using a portable spirometer (Spirolab, MIR Medical International Research SRL, Italy), according to international guidelines (358). Briefly, participants stood with the nose clip attached and the mouthpiece placed in mouth with lips closed tightly around the mouthpiece. Participants then inhaled completely and rapidly with a pause of <1 s at total lung capacity, before exhaling maximally, rapidly and forcefully while maintaining an upright posture for ≥ 6 s until the participant is unable to continue exhaling. An acceptable reading included no coughing or air leakage, no cessation of flow and no extra breath during the procedure. The manoeuvre is then repeated thrice minimally and no more than five times or until the largest values of forced expiratory volume in 1s (FEV1) and forced vital capacity (FVC) were within 0.15 L. Participants were familiarized with the procedure and instructions were explained and demonstrated prior to test. Lung function of participants was determined as the largest absolute values of FVC, FEV1 and the percentage of FEV1/FVC. Lung function was also determined using the predicted values of FEV1, FVC and percentage of FEV1/FVC, which were calculated in the Spirolab software by the ATS/ERS standards, depending on ethnicity group (469).

5.2.5 Arterial stiffness measurement

Arterial stiffness measurement procedures are described in Section 3.2.3 in Chapter 3.

5.2.6 DNA extraction and 16s rRNA gene sequencing

Sputum and saliva samples were thawed on ice and homogenised using glass beads (1mm, Sigma-Aldrich, Missouri, USA) using a bead mill homogeniser (VWR, Pennsylvania, USA). DNA was purified using the Roche High-pure PCR Template Preparation Kit (Roche diagnostics, Rotkreuz, Switzerland). Blank extractions from sterile phosphate buffered saline were also performed and served as negative extraction controls. All extracted samples were quantitated using the Qubit dsDNA High Sensitivity Assay Kit (Invitrogen, Massachusetts, USA) and visually assessed for integrity by electrophoresis on a 0.8% agarose gel.

5.2.7 Sequence data processing and taxonomic assignment

Using extracted sputum DNA samples, libraries for targeted amplicon sequencing were prepared following the “16S Metagenomic Sequencing Library Preparation” guide (Part# 15044223 rev. B, Illumina) (470). This 300-bp paired-end sequencing protocol was performed on a MiSeq sequencing platform (Illumina) at the Lee Kong Chian School of Medicine, Singapore. Targeted amplicon sequences were analysed using the 16S metagenomics tool (version 1.0.1; Illumina), using as a taxonomic database the Illumina-curated version of the May 2013 Greengenes Consortium Database (greengenes.secondgenome.com) release and the Ribosomal Database Project Classifier as the classification algorithm. Control samples from negative PCR and blank DNA extractions were also sequencing and assessed to detect potential contaminants using the decontam statistical package (471).

5.2.8 Statistical analysis

All statistical analyses were performed using Statistical Package for Social Sciences, version 23 (SPSS, Inc., Chicago, IL) and R version 3.3.3 (R Foundation for statistical computing, Vienna, Austria). The effect size for this study cannot be accurately predicted due to the lack

of studies investigating the lung microbiome with age. The estimated total sample size of 48 participants (24 per group) was calculated based on effect sizes observed in other microbiome studies (small effect sizes of <0.1) to achieve a statistical power of at least 0.8 (472). Numerical variables are presented as mean (standard deviation, SD) in text and figures unless otherwise stated. The participant characteristics were analysed using paired t-test to assess potential differences between younger and older groups. Between group differences for relative abundance of both saliva and sputum microbiome phyla and genera, as well as alpha diversity (Shannon, Simpson and inverse Simpson index) were also assessed using paired t-test. Operational taxonomic units were used as input to calculate alpha diversity, and phylogenetic inputs of alpha diversity revealed no difference with taxonomical inputs. Due to the skewed distribution of data, a square root transformation was performed for relative abundances of Firmicutes, Proteobacteria, *Haemophilus* and *Lautropia*, and a logarithmic transformation was performed for PWV to achieve a normal distribution for paired t-test. Pearson's and Spearman's correlation was used to evaluate associations between two variables that were either normally distributed or not normally distributed respectively, including any sub-group analyses. Principal coordinate analysis (PCoA) was used to assess the beta diversity and overall lung microbiome composition with age. PCoA plots for were generated using the first two principal coordinates according to age categories (young vs. elderly), as well as according to sample type (saliva vs. sputum). The 'vegan' R package (version 2.4-5) was used to calculate alpha diversity and implement 'adonis', which uses permutational multivariate analysis of variance, to test for statistical significance of association of overall beta diversity of lung microbiome composition with age. Bray Curtis distances between paired and unpaired young-elderly comparisons were assessed using the "dist_groups" function from the R package 'udist'. All the results in the tables and figures are presented using the non-transformed data. A value of $p < 0.05$ was considered statistically significant.

5.3 Results

5.3.1 Age-associated changes in physiology of study cohort

5.3.1.1 Anthropometry and blood pressure

The demographics of healthy participants in this study are presented in Table 5.1. Despite no difference in weight ($p = 0.734$), older adults were shorter in stature ($p < 0.001$) and had higher BMI ($p = 0.03$) than the younger adults. WC was also greater in older than younger adults ($p = 0.03$). Compared with younger adults, older adults also had higher systolic BP ($p = 0.002$), although diastolic BP was similar between groups ($p = 0.213$) (Table 5.1).

Table 5.1. Mean (SD) of participant demographics, anthropometry, blood pressure, lung function and arterial stiffness parameters

	Young	Elderly	<i>p</i> value
<i>n</i>	24	24	
Sex <i>n</i> (M/F)	11/13	6/18	
Age (years)	29 (5)	63 (2)	<0.001
Weight (kg)	60 (12)	59 (13)	0.734
Height (cm)	166 (9)	157 (9)	<0.001
BMI (kg/m ²)	21.7 (3.0)	23.9 (3.5)	0.03
WC (cm)	72.7 (9.4)	79.5 (11.7)	0.03
Systolic BP (mmHg)	112 (10)	123 (16)	0.002
Diastolic BP (mmHg)	71 (8)	74 (9)	0.213
FEV1 (L)	3.4 (0.7)	2.0 (0.6)	<0.001
FVC (L)	3.9 (1.0)	2.6 (0.6)	<0.001
FEV1/FVC (%)	86 (6)	79 (8)	0.003
FEV1 (%Predicted)	97 (14)	88 (15)	0.063
FVC (%Predicted)	96 (14)	91 (17)	0.361
FEV1/FVC (%Predicted)	100 (7)	97 (10)	0.157
Pulse Wave Velocity (PWV) (m/s)	6.5 (1.1)	8.4 (1.7)	<0.001
Augmentation Index (AIx)	8.7 (9.9)	24.6 (8.1)	<0.001

5.3.1.2 Lung function and arterial stiffness

Lung function parameters including FEV1 ($p < 0.001$), FVC ($p < 0.001$) and FEV1/FVC ($p = 0.003$) were all reduced in older adults compared with their younger family pairs (Table 5.1).

Compared with younger adults, older adults had 41% or 1.4 L lower FEV1, 33% or 1.3 L lower FVC and 7% lower percentage of FEV1/FVC. However, adjusted FEV1 (%Predicted) and FVC (%Predicted) were comparable between younger and older adults ($p = 0.063$ and $p = 0.361$). The lower values of lung function parameters were reflective of natural lung function decline in the healthy elderly cohort. The percentage of predicted value for FEV1/FVC percentage in younger and older family pairs was comparable ($p = 0.157$), and the values were all within healthy ranges (473). Compared with younger adults, older adults had increased arterial stiffness, indicated by arterial stiffness parameters PWV and AIx (all $p < 0.001$). In both younger and older adults, arterial stiffness parameters PWV and AIx were also within healthy ranges (390), and were 1.3-fold and 3.9-fold higher in older than in younger adults respectively (Table 5.1). Lung function parameters were inversely associated with arterial stiffness (Table 5.2). However, when age was adjusted for, in a partial correlation model, the associations between FEV1, FVC and percentage FEV1/FVC, with arterial stiffness parameters AIx and PWV were not significant (Table 5.2).

Table 5.2. Correlation matrix indicating associations between lung function and arterial stiffness parameters, with and without adjustment for age in a partial correlation model. Correlation coefficients and corresponding p values are shown in brackets.

	Without age adjustment		With age adjustment	
	AIx	PWV	AIx	PWV
FEV1 (L)	-0.519 (<0.001)***	-0.396 (0.008)**	-0.043 (0.786)	0.098 (0.531)
FVC (L)	-0.509 (<0.001)***	-0.321 (0.033)*	-0.108 (0.492)	0.165 (0.290)
FEV1/FVC (%)	-0.247 (0.106)	-0.508 (<0.001)***	0.194 (0.212)	-0.158 (0.313)
FEV1 (%Predicted)	-0.182 (0.237)	-0.118 (0.445)	-0.017 (0.913)	-0.027 (0.865)
FVC (%Predicted)	-0.129 (0.404)	0.064 (0.681)	-0.121 (0.439)	0.034 (0.831)
FEV1/FVC (%Predicted)	-0.054	-0.253	0.190	-0.099

	(0.727)	(0.098)	(0.223)	(0.526)
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* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.3.2 Age-associated changes in lung microbiome composition

5.3.2.1 Composition of lung microbiome from sputum samples in healthy individuals

The main phyla and genera identified in the airway microbiome of younger and older adults are shown in Figure 5.1. Sputum samples from healthy individuals, both young and elderly, consisted mainly of microbes from phylum Firmicutes (46%), Proteobacteria (16%), Bacteroidetes (16%), Actinobacteria (14%) and Fusobacteria (6%). The main genera detected were *Streptococcus* (24%), *Prevotella* (12%), *Neisseria* (10%), *Rothia* (8%), *Veillonella* (7%), *Granulicatella* (4%), *Fusobacterium* (4%), *Actinomyces* (3%), *Gemella* (2%), *Haemophilus* (2%), *Porphyromonas* (2%), *Leptotrichia* (2%), *Campylobacter* (1%) and *Lautropia* (1%) (Figure 5.1A). Comparison of saliva samples (total $n = 6$) suggested a high degree of overlap between the microbiota of healthy sputum and saliva with no differences in alpha- (Shannon diversity index (SDI) $p = 0.304$) or beta-diversity ($p = 0.582$) (Figure 5.2). However, sputum samples, which are commonly used to represent the lung microbiome, exhibited subtle differences in specific taxa including higher relative abundance of *Granulicatella* (4.3% vs. 3.0%, $p = 0.031$), lower relative abundance of *Leptotrichia* (0.8% vs. 1.4%, $p = 0.013$) and lower relative abundance of *Corynebacterium* (0.7% vs. 0.1%, $p = 0.031$) (Figure 5.2).

Figure 5.1. Lung microbiome composition in genetically paired young and elderly subjects. Lung microbiome composition as detected by 16s rRNA gene profiling (A) Average relative abundance (RA) for the most representative taxa (present at >1%) is illustrated by circle size, colour denotes phylum level membership. Principle co-ordinate analysis (PCoA) of bray-curtis distance between microbiome profiles observed in young (red triangles) and elderly (turquoise circles) cohorts (B). PCoA of microbiome profiles indicating linked young (triangles) and elderly (circles) participant pairs. Grey lines illustrate the distance between each paired sample which also share the same colour (C). Comparison of bray-curtis distance between PCoA points illustrating the greater similarity observed between paired, genetically-related participants (D). * = $p < 0.05$.

Figure 5.2. Analysis of microbiome profiles of saliva and sputum samples. Comparison of lung microbiome composition as detected by 16S rRNA gene profiling in DNA (A). Average relative abundance (RA) for the most representative taxa (present at >1%) is illustrated by circle size, colour denotes phylum level membership. PCoA of Bray-Curtis distance (B), Shannon diversity index (C), and differences in relative abundance of *Granulicatella* (D), *Leptotrichia* (E) and *Corynebacterium* (F) between saliva (V) and sputum (P) samples of 3 young and elderly family pairs. * = $p < 0.05$

5.3.2.2 Comparison of lung microbiota present in genetically related young and elderly family pairs

In order to allow some degree of control for genetic confounders, this study assessed the airway microbiota in related young-elderly family pairs. PCoA analysis revealed that the sputum microbiome of younger and older adults was more similar when analysed as matched family pairs. Here, the measured Bray Curtis distance of matched young-elderly pairs was lower (equating to more similar microbiome profiles) when compared to the average distance between all other possible un-matched young-elderly comparisons ($p = 0.036$, Figure 5.1C, 5.1D). Paired analysis showed that older adults generally had higher relative abundance of Firmicutes (47.4% vs 43.9%, $p = 0.035$) and lower Proteobacteria (13.9% vs. 19.0%, $p = 0.014$) with lower average relative abundance of Proteobacteria including *Haemophilus* (2.0% vs. 2.8%, $p = 0.024$) and *Lautropia* (0.5% vs. 1.1%, $p = 0.020$) (Figure 5.3). No significant differences in alpha diversity measures were observed between young and elderly groups (Figure 5.4). Beta-diversity analysis also showed no difference between age groups in overall lung microbiome composition ($p = 0.420$, Figure 5.1B).

Figure 5.3. Differentially abundant taxa in sputum samples of genetically paired young and elderly subjects. At the phylum level, elderly adults have higher relative abundance of Firmicutes (A), and lower relative abundance of Proteobacteria (B) than young adults. At the genus level, elderly adults have lower relative abundances of *Haemophilus* (C) and *Lautropia* (D). Black connecting lines indicate young-elderly pairs. * = $p < 0.05$.

Figure 5.4. Alpha diversity indexes between genetically paired young and elderly subjects. No significant differences are observed in Shannon diversity index (A) Simpson index (B) and Inverse Simpson index (C) between young (white bars) and elderly (grey bars) paired groups. Black connecting lines indicate young-elderly pairs. ns = not significant

5.3.3 Association of lung microbiome with lung function and arterial stiffness

5.3.3.1 Association of lung microbiome alpha diversity with lung function and arterial stiffness

Alpha diversity of the lung microbiome was associated with lung function in an age-dependent manner. While SDI was associated with both FEV1 (%Predicted) and FVC (%Predicted) in the cohort as a whole (all $r = -0.314$, $p = 0.032$), the association was driven by the younger group (Figure 5.5A, B). In the younger group, FEV1 (%Predicted) and FVC (%Predicted) were both associated with SDI ($r = -0.546$, $p = 0.006$ and $r = -0.573$, $p = 0.003$) (Figure 5.5A, B). However, in the older group, the association between FEV1 (%Predicted) and FVC (%Predicted) with SDI were not significant ($r = -0.155$, $p = 0.481$ and $r = -0.086$, $p = 0.696$) (Figure 5.5A, B). In the young group, SDI was also associated with absolute FVC ($r = -0.411$, $p = 0.031$) (Table 5.3). Conversely, alpha diversity of the lung microbiome was correlated with arterial stiffness (PWV) in the older but not the younger group ($r = -0.516$, $p = 0.01$ vs $r = 0.132$, $p = 0.569$) (Figure 5.5C). Correlations with inverse Simpson index showed similar associations as SDI (Table 5.3).

Figure 5.5. Sub-group correlations between microbiome alpha-diversity with lung function and arterial stiffness in young and elderly subjects. Correlation between Shannon diversity index of the lung microbiome and lung function parameters FEV₁ (%Predicted) (A), FVC (%Predicted) (B) and arterial stiffness parameter pulse wave velocity (C). The line of best fit is indicated with a broken line for the young population and with a continuous line for the elderly. Correlation coefficients and associated p values are indicated for the total population (r_{total}), the young sub-group (r_{young}) and the elderly sub-group (r_{elderly}).

Table 5.3. Correlation matrix comparing lung microbiome composition with lung function and arterial stiffness parameters within young and elderly adults.

		FEV1	FVC	FEV1 (%pred)	FVC (%pred)	AIx	PWV
Young	SDI	-0.362 (0.082)	-0.411 (0.031)*	-0.546 (0.006)**	-0.573 (0.003)**	-0.068 (0.769)	0.132 (0.569)
	ISI	-0.396 (0.055)	-0.460 (0.024)*	-0.440 (0.032)*	-0.482 (0.017)*	0.009 (0.967)	0.169 (0.465)
	Firmicutes	0.318 (0.130)	0.390 (0.060)	0.363 (0.081)	0.409 (0.047)*	0.159 (0.492)	0.160 (0.489)
	Gemella	0.082 (0.704)	0.017 (0.937)	-0.348 (0.095)	-0.422 (0.040)*	-0.072 (0.756)	-0.080 (0.732)
	Actinobacteria	-0.055 (0.799)	-0.070 (0.747)	0.064 (0.767)	0.023 (0.916)	0.019 (0.935)	-0.121 (0.603)
	Actinomyces	-0.408 (0.048)*	-0.391 (0.059)	-0.198 (0.354)	-0.142 (0.508)	-0.119 (0.608)	0.205 (0.372)
	Fusobacteria	-0.151 (0.480)	-0.168 (0.432)	-0.395 (0.056)	-0.364 (0.080)	0.014 (0.953)	0.146 (0.528)
	Leptotrichia	-0.132 (0.538)	-0.112 (0.603)	-0.353 (0.090)	-0.216 (0.310)	0.012 (0.960)	0.170 (0.461)
	Lautropia	0.043 (0.843)	-0.124 (0.563)	0.015 (0.944)	-0.209 (0.327)	-0.492 (0.023)*	-0.420 (0.058)
Elderly	SDI	-0.151 (0.493)	-0.157 (0.475)	-0.155 (0.481)	-0.086 (0.696)	0.035 (0.870)	-0.516 (0.010)*
	ISI	-0.216 (0.323)	-0.206 (0.346)	-0.112 (0.611)	-0.017 (0.939)	0.055 (0.789)	-0.568 (0.004)**
	Firmicutes	0.190 (0.386)	0.099 (0.653)	-0.031 (0.888)	-0.200 (0.360)	0.193 (0.366)	0.118 (0.582)
	Gemella	-0.259 (0.232)	-0.215 (0.324)	-0.032 (0.887)	0.082 (0.710)	0.124 (0.564)	0.280 (0.186)
	Actinobacteria	0.347 (0.105)	0.447 (0.033)*	0.382 (0.072)	0.322 (0.134)	0.137 (0.522)	0.097 (0.651)
	Actinomyces	0.230 (0.290)	0.238 (0.274)	0.124 (0.572)	0.073 (0.742)	0.023 (0.915)	-0.350 (0.094)
	Fusobacteria	-0.361 (0.091)	-0.344 (0.108)	-0.286 (0.187)	-0.114 (0.605)	0.083 (0.701)	-0.570 (0.004)**
	Leptotrichia	-0.284 (0.189)	-0.221 (0.310)	-0.318 (0.139)	-0.157 (0.473)	0.207 (0.332)	-0.569 (0.004)**
	Lautropia	-0.088 (0.690)	-0.112 (0.612)	0.254 (0.242)	0.080 (0.716)	0.034 (0.873)	-0.093 (0.665)

Microbiome indices of alpha diversity including the Shannon diversity index (SDI) and inverse Simpson index (ISI) as well as specific phyla and genera showing significant correlation with lung function (FEV1, FVC, FEV1 %Predicted, FVC %Predicted) and arterial stiffness (augmentation index (AIx) and pulse wave velocity (PWV)) are displayed for younger and elderly sub-groups. * $p < 0.05$, ** $p < 0.01$). p values are shown in brackets.

5.3.3.2 Age-dependent association of lung microbiome composition and lung function

Within the younger cohort, the relative abundance of microbes from the Firmicutes phylum ($r = 0.409, p = 0.047$) and specifically the *Gemella* genus ($r = -0.422, p = 0.04$) was associated with FVC (%Predicted) (Figure 5.6A, B). However, similar to correlations with SDI, these associations between FVC (%Predicted) were seen in the younger but not the elderly cohort ($r = -0.200, p = 0.360$ and $r = 0.422, p = 0.710$) (Figure 5.6A, B). In younger subjects, the relative abundance of *Actinomyces* genus was also inversely associated with absolute FEV1 but this association was not observed among older individuals ($r = -0.408, p = 0.048$ vs. $r = 0.230, p = 0.290$) (Table 5.3). Within the older group, the relative abundance of phylum Actinobacteria was positively associated with absolute FVC ($r = 0.447, p = 0.033$), an association that was not observed in the younger group ($r = -0.070, p = 0.747$) (Figure 5.6C).

Figure 5.6. Sub-group correlation between specific lung microbiota with lung function and arterial stiffness parameters. In the young but not the elderly group, FVC (%Predicted) correlated with relative abundance of phylum Firmicutes (A) and genus *Gemella* (B). FVC correlated with relative abundance of genus *Actinobacteria* in the elderly but not the young group (C). In the elderly, arterial stiffness and lung microbiome correlations were observed between pulse wave velocity and relative abundance of phylum Fusobacteria (D) and genus *Leptotrichia* (E). In the young, augmentation index correlated with relative abundance of *Lautropia* (F). Young and elderly measures are indicated by unfilled and filled circles respectively. The line of best fit is indicated with a broken line for the young population and with a continuous line for the elderly. Correlation coefficients and associated p values are indicated for young sub-group (r_{young}) and elderly sub-groups (r_{elderly}).

5.3.3.3 Age-dependent association of lung microbiome composition and arterial stiffness

In contrast to the associations between lung microbiome and lung function parameters, which were more prevalent in the younger group, the associations between lung microbiome and arterial stiffness were more pronounced in the older group. Within the older group, the arterial stiffness parameter PWV exhibited an inverse association with relative abundances of the Fusobacteria ($r = -0.570, p = 0.004$), specifically the genus *Leptotrichia* ($r = -0.569, p = 0.004$) (Figure 5.6D, E). These associations of PWV with both Fusobacteria and *Leptotrichia* relative abundances were not significant in the younger group ($r = 0.146, p = 0.528$ and $r = 0.170, p = 0.461$) (Figure 5.6D, E). The only genus exhibiting correlation with arterial stiffness in the young was *Lautropia*, which was associated with AIx an observation that was not evident in the older cohort ($r = -0.492, p = 0.023$ vs $r = 0.034, p = 0.873$) (Figure 5.6F). These age-specific associations between airway microbiome composition, lung function and arterial stiffness are summarised in Figure 5.7.

Figure 5.7. Summary schematic showing the significant associations between lung microbiome with lung function and arterial stiffness, within the young and elderly groups. Lung microbiome is associated with lung function mostly in the young group, as Shannon diversity, Firmicutes phylum and its genus *Gemella* are associated

with predicted lung function, while absolute FEV1 is associated with *Actinomyces* (in grey). In the elderly group, absolute FVC is associated with Actinobacteria (in grey). Lung microbiome is associated with arterial stiffness mostly in the elderly group, with Shannon diversity, Fusobacteria phylum and its genus *Leptotrichia* associated with pulse wave velocity. In the young group, *Lautropia* is associated with augmentation index parameter of arterial stiffness.

5.4 Discussion

While there is a significant increase in the number of studies on ageing gut microbiome and its consequence for health and disease, comparable analyses of the respiratory microbiome are lacking (33, 260, 474). Although several studies have investigated the airway microbiome in the context of respiratory disease, the present study is the first to explore age-associated differences in lung microbiome composition between family pairs, with specific focus on healthy individuals. This present finding demonstrates consistency between young and elderly healthy lung microbiome profiles but with subtle taxonomic differences. The lung microbiome was independently associated with lung function (FEV1 %Predicted and FVC %Predicted) in the younger adults, and with arterial stiffness parameter (PWV) in the elderly, suggesting that these relationships have greater relevance at different stages of life.

To my knowledge, this study is the first to investigate healthy airway microbiome in young and older family pairs to date and demonstrated that several phyla, including Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria and Fusobacteria, define the healthy airway. These observations were in agreement with earlier studies implicating six predominant phyla of Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria and Cyanobacteria as the major constituents of the human microbiome at the skin and gut, and is consistent with studies of the oral microbiome in healthy individuals (475, 476). While no studies to date have directly investigated the airway microbiome in healthy individuals, other investigations comparing healthy controls with the asthmatic lung microbiome have shown similar core

human lung microbiome in healthy people, including *Streptococcus*, *Prevotella*, *Neisseria*, *Veillonella*, *Haemophilus* and *Fusobacterium* genera (38, 477). In lung diseases such as asthma, there is a shift in lung microbiota towards Proteobacteria (mainly *Haemophilus*), Firmicutes (mainly *Streptococcus*), Bacteroidetes (mainly *Prevotella*) and Actinobacteria (38). Although associations can be drawn between disease states and lung microbiome, the function and causal role of the microbiome in lung diseases remain unclear and studies of the lung microbiome in healthy individuals are lacking. Therefore, the profile of microbiome found in this study represents an important reference and starting point for characterising the lung microbiome in healthy individuals, which can be useful for future research on lung microbiome.

Though long-speculated to be of major clinical significance, the role of the airway microbiome in ageing is still unknown (478). In this study, there was no difference in lung microbiome diversity between younger and older family pairs. While the microbiome diversity in the gut increases with age in healthy people across different populations, the increase in diversity seem to occur largely from 0–3 years old and remained fairly constant throughout adulthood (479). In this study, younger adults were 21–39 years old and the changes in the lung microbiome may be less obvious compared with older adults. Although older adults are more susceptible to lung diseases, which are associated with a loss of lung microbial diversity (480), the older adults in this study were all healthy individuals free from respiratory diseases. It is possible that the specific bacteria composition rather than the microbial diversity is altered with ‘healthy’ ageing. Compared with younger adults, the lung microbiome of older adults in this study was associated with lower relative abundance of the bacterial OTU from phylum Proteobacteria (*Haemophilus* and *Lautropia*) and greater relative abundance of the Firmicutes. In patients with COPD, sputum and lung tissue samples had an overrepresentation of the Proteobacteria and a reduction in Firmicute abundance than non-COPD controls (481–483). Enrichment of specific

Proteobacteria, such as *Haemophilus* in the sputum microbiome of patients with COPD (484) and *Lautropia* in cystic fibrosis patients, may represent examples of disease-associated dysbiosis (483, 485). However, in the present study, healthy individuals had an overall representation of Proteobacteria (16%), which was much lower than in diseased states (~44%) (481). In addition, the average Firmicutes representation (46%) in this study was higher compared to diseased states (~16%) (481). It is plausible that 'healthy' ageing of the lungs promotes enrichment of the Firmicutes and reduced relative abundance of Proteobacteria such as *Haemophilus* and *Lautropia*, which contrasted with the dysbiosis associated with respiratory diseases.

The correlation between lung microbiome composition and lung function was observed in younger adults, with an inverse relationship between lung microbiome alpha diversity (SDI) and lung function (FEV1 %Predicted and FVC %Predicted). In contrast, in patients with COPD, reduced microbial diversity was associated with impaired lung function, due to predominance of pathogenic Proteobacteria, such as *Haemophilus* and *Pseudomonas* and a relative decrease in the Firmicutes (481, 483, 486). At the phylum level, Firmicutes abundance was positively associated with lung function (FVC %Predicted) in the young. These results suggest that Firmicutes may have beneficial effects on lung function and might explain the negative association between lung function and SDI, as their increased abundance may drive a reduction in diversity. The observed association between reduced lung function and increased relative abundance of *Gemella* (a Firmicute linked to pulmonary exacerbation in cystic fibrosis patients) in young adults suggests the potential negative implications for respiratory health (270). Taken together, these results suggest that the positive relationship between Firmicute abundance and lung function may be contingent on the presence and abundance of specific genera within this phylum, such as *Gemella*. In the present study, Actinobacteria was positively associated with

absolute FVC in older adults and *Actinomyces* was negatively associated with absolute FEV1 in younger adults. However, absolute values of FEV1 and FVC are less robust lung function parameters that do not take into consideration normative physiological lung function decline with age. Therefore, the association between lung microbiome and lung function (FEV1 and FVC %Predicted) may be more relevant and most pronounced in the younger group (Figure 5.7).

In contrast to lung function, associations between airway microbiome composition and arterial stiffness were largely confined to the older group in this study. Lung microbiome diversity (SDI) was inversely associated with arterial stiffness (PWV), in the older but not in the younger adults in this study. Consistent with these findings, loss of lung microbial diversity was associated with severe lung diseases, likely due to increased dominance of pathogenic microbes (480). Reduced microbial diversity in lower airways was linked to inflammatory phenotypes of the airways (487-489), which could increase both systemic inflammation and arterial stiffness that occur during ageing (39, 484). For example, a decrease in lung bacterial diversity was associated with increased alveolar concentrations of pro-inflammatory and pro-fibrotic cytokines in humans (179). Increased inflammatory state in the airway was also associated with increased arterial stiffness (417, 418), suggesting that the lower lung microbiome diversity in the older adults may be associated with mild inflammatory states that could increase arterial stiffness. The relative abundance of Fusobacteria (*Leptotrichia*) were inversely associated with arterial stiffness in older adults in this study, and an increase in the relative abundance of Fusobacteria was associated with decreased risk of lung cancer amongst non-smokers (490, 491). Also, increased relative abundance of Fusobacteria and *Leptotrichia* in the oral microbiome were both associated with decreased risk of pancreatic cancer (492), highlighting the potential protective functions of these organisms. In younger adults, the relative abundance

of *Lautropia* was inversely associated with the alternative arterial stiffness parameter AIx. *Lautropia* was present in bronchoalveolar lavage of clinically stable but not in exacerbating bronchiectasis patients (493), which suggests that *Lautropia* might have protective functions. The differential associations between arterial stiffness and lung microbial organisms in the younger and older adults could be due to different measures of arterial stiffness. Unlike PWV which measures static properties such as distensibility, AIx measures the dynamic properties including the endothelial function and peripheral resistance (494). These results suggest that specific microbes in the lungs plausibly influence different components of arterial stiffness in an age-dependent manner (Figure 5.7).

Reduced lung function has been implicated in arterial stiffness (41). In this study, arterial stiffness was negatively associated with lung function, which was not statistically significant after adjusting for age. In contrast to my findings, in healthy normal weight Chinese and European individuals, lung function was inversely associated with arterial stiffness, even after adjusting for age and height (227-229). In people with COPD, FEV1/FVC ratio was also independently and negatively associated to arterial stiffness (230, 491, 495). While the mechanism underlying this established association is not known, a link to systemic inflammation has been proposed (496). The differences in findings are likely due to the study design, as this study recruited participants in groups (younger ≤ 40 years and older ≥ 60 years), which introduced age as a confounder of correlation analysis. Participants between 40–60 years old were omitted in this study and may have obscured age-dependent correlation between lung function and arterial stiffness.

This study had several limitations. Although associations could be drawn from the study results, the cross-sectional design did not conclusively prove causality. The correlations reported in

this study were associative and explorative and did not shed light on causality between exercise habits, ageing and lung microbiome. Future studies involving larger sample sizes are warranted to better appreciate the associations between the microbial taxa with lung function and arterial stiffness. This study was also unable to directly assess temporal dynamics of the lung microbiome during ageing, given the lack of a longitudinal component. While the younger and older participants in this study were family pairs, environmental factors (lifestyle or living conditions) could have influenced the lung microbiome, which were not accounted for in the present study design. Lung microbiome of family pairs who do not live together or share the same diet may be influenced by these environmental factors. However, the matched family pair study design showed a more similar lung microbiome profile than the un-matched young–elderly adults, suggesting some genetic influence on the lung microbiome which should be accounted for in studies investigating lung microbiomes. As targeted bacterial 16s rRNA gene amplicon sequencing was employed, the resolution does not support species-level characterisation of the microbiome, which could be important in understanding the ecological and functional interaction with ageing. However, though explorative in nature, this study provided early and novel insights into age-dependent variability in the airway microbiome and its potential implications for respiratory and cardiovascular health. This study identified several potential microbial taxa whose clinical relevance should be further explicated in larger studies incorporating longitudinal experimental design to fully delineate the core healthy airway microbiome and the temporal dynamics of protective and deleterious taxa that may serve as early prognostic indicators of lung health.

In conclusion, this study characterised for the first time, the airway microbiome of healthy individuals and found specific age-associated changes in their microbiome profiles. This study

proposed that different organisms may independently influence lung function and arterial stiffness in an age-dependent manner.

Chapter 6: Summary and conclusion

The primary aim of this thesis was to investigate the associations of fibroblast growth factor 21 (FGF21) with the beneficial effects of ageing and exercise habits on glucose regulation, arterial stiffness and bone mass. This thesis also explored age-associated changes in airway microbiome and its associations with physiological and health status in young and older family pairs. Ageing is associated with various chronic health conditions such as the decline in glucose regulation, bone mass and lung function and the increase in arterial stiffness (8-10). Conversely, exercise training is associated with improvements in glucose regulation, lung function, arterial stiffness and bone mineral density (BMD) (18). Current evidence suggests that FGF21 may play important roles in improving glucose regulation, increasing bone resorption and in anti-oxidative and cardioprotective functions (51, 276, 409). This evidence suggests the potential associations between FGF21 with age- and exercise-related effects on glucose regulation, arterial stiffness and bone mass.

Three studies were conducted to investigate the associations of FGF21 on the effects that exercise and age, have on glucose regulation, bone mass and density, and arterial stiffness (measured indirectly with pulse wave velocity). A fourth study explored and characterised the microbiome in the lungs of young and older family pairs and their associations with physiological functions and health indicators. The key findings in this thesis are:

- a. FGF21 is associated with age- and habitual exercise-related glucoregulatory changes. FGF21 concentration during an oral glucose tolerance test (OGTT) differed with age and with habitual exercise. However, adiponectin concentration during the OGTT was not affected by age or habitual exercise. Contrary to current consensus, the effects of FGF21 on glucose regulation was not associated with adiponectin.

- b. Oxidative stress is related to the age-associated increase in arterial stiffness, but unlikely through FGF21. Oxidized low-density lipoprotein (OxLDL) concentration and arterial stiffness increased with age, but FGF21 concentration was not affected by age. While the anti-oxidative properties of FGF21 were shown in animals and cell models, FGF21 is not associated with oxidative stress in healthy humans, which suggests that the anti-oxidative effects of FGF21 observed in animals might not be translatable to humans.
- c. FGF21 concentration, concentration of bone turnover markers and BMD increased with age, suggesting that FGF21 may contribute to age-associated decline in BMD. However, insulin-like growth factor binding protein 1 (IGFBP1) concentration was not affected by age, implying that the suggested FGF21 downstream effector IGFBP1 shown in animal models is unlikely involved in healthy humans. Other potential FGF21 downstream regulators associated with oxidative stress or peroxisome proliferator-activated receptor (PPAR)- γ -pathways might be involved.

This thesis also explored, for the first time, the effects of ageing on lung microbiome and the associations of lung microbiome with arterial stiffness and lung function in young and elderly family pairs. In healthy individuals, ageing was associated with an increase in relative abundance of Firmicutes and a decrease in relative abundance of Proteobacteria, and different organisms may independently influence lung function and arterial stiffness in an age-dependent manner. While most studies have investigated the role of the gut microbiome in ageing, this study represents an important starting point for characterising the lung microbiome in healthy individuals, which is an integral reference point before disease onset.

The findings of this thesis are unique to clinically healthy individuals, which is an important reference point in understanding the underlying mechanisms for chronic disease progression, without the influence of existing chronic disease. Most of the earlier studies have investigated the role of FGF21 and the lung microbiome in diseased states, which do not reflect normative changes in physiology. It is important to identify the underlying mechanisms that drive physiological changes that occur with exercise or during normative ageing, as these mechanisms are key to early intervention before disease onset.

6.1 Associations of FGF21 with exercise, ageing and glucose regulation

The primary aim of study 1 was to investigate the association between FGF21 and glucose regulation, under the influence of exercise habits and ageing in healthy individuals. This study compared the response of blood glucose, plasma insulin, FGF21 and adiponectin concentrations in response to an oral glucose tolerance test (OGTT) between younger (18–36) and older (45–80) individuals. Within each age group, the participants were matched for sex and age in 5-year intervals and assigned to habitually active and sedentary subgroups based on their exercise history. Under the OGTT procedure, habitual exercise was associated with lower FGF21 and insulin concentrations regardless of age, and ageing was independently associated with increased glucose and FGF21 concentrations regardless of exercise habits. Adiponectin response during the OGTT was not associated with both ageing and habitual exercise. The acute response of FGF21, but not adiponectin, to the oral glucose load differed with age and with exercise habits, suggesting that FGF21 modulated the associations between ageing and habitual exercise on glucose regulation. However, contrary to current evidence in animal models and in people with type 2 diabetes mellitus (T2DM) (48, 302, 329), my study on healthy people found that adiponectin may not participate in the FGF21 pathway associated with the effects of ageing and habitual exercise on glucose regulation. This finding that suggests there

may be different pathways regulating the effects of exercise and ageing on glucose regulation. Although FGF21 may be associated with the effects of exercise and ageing on glucose regulation in both healthy people and T2DM patients, adiponectin is only active in the presence of T2DM. Since adiponectin is an adipokine, the passive response of adiponectin in healthy people may be due to normal body mass index status in this group, which contrasted with the positive association of adiponectin with glucose regulation commonly reported between obesity and T2DM states (497). This finding addresses a critical gap in identifying FGF21 as a mechanism that drives the decline in glucose regulation during normative ageing in people with normal glucose tolerance, and may potentially contribute to early stratification of metabolic risk with age. The identification of FGF21 involvement in habitual exercise-associated improvement in glucose regulation also provides insights for the development of exercise mimetics or therapeutic targets for T2DM.

The hypothesis that glucose regulation with habitual exercise and ageing are associated with FGF21 is supported by the results of this study.

6.2 FGF21 unlikely attenuates oxidative stress and associated arterial stiffness with ageing and exercise

The primary aim of study 2 was to investigate the FGF21-associated pathways on arterial stiffness, under the influence of ageing and habitual exercise in healthy individuals. The secondary aim was to explore the associations of FGF21 with oxidative stress and arterial stiffness. This study compared plasma concentrations of FGF21, adiponectin and oxLDL (a marker for oxidative stress) and arterial stiffness between younger (18–36) and older (45–80) groups, and between habitually active and sedentary subgroups within each age group. Oxidative stress and arterial stiffness were higher in older than younger adults, independent of

exercise habits, despite no difference in FGF21 and adiponectin concentrations. These results support the previous finding that age-associated increase in arterial stiffness is mediated by oxidative stress (190), independent of the FGF21–adiponectin pathways. Contrary to current consensus on the health benefits of exercise (204, 205), both oxidative stress and arterial stiffness were not different between habitually active and sedentary individuals, regardless of age. These results imply that self-reported exercise habits may not be effective in differentiating the risk of oxidative stress-associated arterial stiffness in healthy individuals. However, FGF21 and adiponectin concentrations were independently associated with arterial stiffness in the sedentary but not in the active group, implying that FGF21 and adiponectin may influence arterial stiffness independently in sedentary individuals. Contrary to the proposed anti-oxidative function of FGF21 reported in cell and animal models (47, 322), my study found that FGF21 had no effect on oxidative stress in healthy humans. This finding implies that while FGF21 might be a potential therapeutic target for abnormal glucose regulation during ageing, its anti-oxidative properties in attenuating age-associated arterial stiffness is less clear in healthy humans.

The hypothesis that ageing is associated with an increase in oxidative stress and arterial stiffness is supported but the hypothesis that habitual exercise is associated with a decrease in oxidative stress-induced arterial stiffness is defeated. The hypothesis that FGF21 is related to the oxidative stress and arterial stiffness is not supported by this study.

6.3 Association of FGF21 with age-related decline in BMD

The aim of study 3 was to investigate the interaction of FGF21, bone turnover and BMD between healthy younger and older family pairs. Serum concentrations of FGF21, IGFBP1, receptor activator of nuclear factor kappa-B ligand (RANKL), bone formation marker bone-

specific alkaline phosphatase (BAP) and bone resorption marker tartrate-resistant acid phosphatase 5b (TRAP5b), and BMD were measured in 20 pairs of younger and older family members. FGF21 concentration was higher in older than younger adults despite no difference in IGFBP1 concentration. Compared with younger adults, older adults had lower BMD and serum RANKL concentration and higher serum concentrations of TRAP5b and BAP. These results imply that FGF21 may contribute to age-associated decline in BMD through an increase in bone turnover. Although ageing was associated with lower RANKL concentrations, the concentrations of bone formation and bone resorption markers increased with ageing. These results imply an imbalance in bone turnover during ageing, which favours osteoclast bone resorption over osteoblast bone formation, resulting in bone loss and decline in BMD (77). Results from the present study suggests that in healthy humans, IGFBP1 may not be a downstream effector of FGF21 in driving age-associated decline in BMD and in RANKL-associated osteoclast differentiation. These results support the notion that increasing FGF21 concentration during ageing may affect bone remodelling, which is an integral process in healthy ageing. New knowledge on potential key regulators in age-associated bone loss in healthy humans will provide key insights into disease pathology as well as better treatment.

The results from this study supported the hypothesis that FGF21 is involved in age-associated decline in BMD but defeated the hypothesis that IGFBP1 is involved in the FGF21-regulatory axis.

6.4 Age-associated changes in lung microbiome profiles are independently linked to lung function and arterial stiffness.

The primary aim of study 4 was to explore age-associated changes in lung microbiome and their links with arterial stiffness and lung function in healthy younger and older family pairs.

Bacterial microbiome composition was determined from sputum samples collected from 24 pairs of younger and older family members, who also had their arterial stiffness and lung function measured. Ageing was associated with increased arterial stiffness, decreased lung function parameters (forced expiratory volume in 1s [FEV1], forced vital capacity [FVC] and FEV1/FVC percentage), increased bacteria relative abundance from the Firmicutes phylum and decreased relative abundance from the Proteobacteria phylum. Specific lung microbial taxa were associated with lung function parameters (FEV1 %Predicted and FVC %Predicted) in the younger group, with a decrease in alpha diversity and an increase in relative abundance of Firmicutes associated with greater lung function. Separate microbial taxa were associated with arterial stiffness in the older group, with lower alpha diversity and relative abundance of Fusobacteria associated with increased arterial stiffness. These results suggest that ageing is associated with lung microbiome changes in healthy individuals, and that different microbes in the lungs may independently influence lung function and arterial stiffness in an age-dependent manner. This study provided preliminary evidence for the core airway microbiome in healthy individuals and its beneficial and deleterious taxa that may serve as early prognostic indicators of disease.

The hypothesis that the age-associated changes in lung microbiome composition is related with changes in lung function and arterial stiffness during ageing is supported.

6.5 Implications for FGF21 and the lung microbiome in age-associated chronic diseases

Current research on FGF21 focuses on elucidating its mechanistic pathways in improving metabolic diseases and impacting bone fragility in animal models, and the potential therapeutic roles of FGF21 in diseases such as T2DM (51, 276). However, there is limited research data on the associations of FGF21 with normative ageing and age-associated decline in

physiological function and health in healthy individuals, including glucose regulation, arterial stiffness and bone mass. The results from this thesis imply that FGF21 may have opposing effects on health, in promoting glucose regulation and bone loss. These results represent the early evidence on the relationships of FGF21, glucose regulation and BMD and highlighted the importance to further understand the “trade-off” effects of FGF21 and whether an optimal FGF21 concentration or dosage exist. These research questions can be further studied with administration of varying doses of FGF21 in animals to observe its effects on glucose regulation and bone mass, especially in the context of ageing. A deeper understanding on these functions of FGF21 is important to promote early intervention for disease prevention, especially in higher risk groups, and to mitigate or delay the onset of these health conditions with age.

While the gut microbiome has been suggested to be involved in a multitude of age-associated chronic diseases, the association of the lung microbiome with age is not well-investigated. Present studies on the lung microbiome focuses on the associations with various respiratory diseases, but studies in healthy humans are lacking. Lung microbiome and FGF21 data from healthy individuals provide an important pre-disease baseline and reference point for addressing the mechanisms of FGF21 and the lung microbiome, as there are no confounding disease factors that may affect the effects of FGF21 and the lung microbiome on ageing.

The results from this thesis suggest that in healthy individuals, FGF21 may be related to the age-associated decline in glucose regulation and the loss in bone mass. FGF21 is also associated with the positive effects of habitual exercise on glucose regulation amongst healthy individuals in this study, but my results disagreed with earlier findings on animals and people with T2DM that adiponectin is involved as a downstream regulator of FGF21(338). FGF21-

associated pathways with ageing and exercise on glucose regulation may differ between healthy individuals and T2DM patients. The finding that adiponectin is not associated with FGF21 in healthy individuals during normative ageing is novel and adds to the understanding of age-related decline in physiological function in pre-disease state. These results on a cross-section of younger and older healthy individuals offered an important baseline for understanding the risks and drivers of age-associated chronic disease risk. FGF21 is also a plausible mediator of the favourable multi-potent and anti-ageing effects of exercise, which raises the potential of FGF21 as a biomarker for exercise mimetic research.

The exploratory results of specific age-associated changes in microbial taxa in the lungs provided an early-stage characterisation of the lung microbiome in healthy younger and older family pairs, which controlled for the effects of genetic predisposition and disease. This information and their associations with physiological and health indicators are important reference for future research on the relationship between airway microbiome and ageing health. Since arterial stiffness is an underlying mechanism that contributes to the pathology of a multitude of age-associated chronic diseases (14), the associations between the lung microbiome with lung function and arterial stiffness suggest that the lung microbiome may be involved in the age-associated chronic diseases. The associations between the lung microbiome and normative decline in physiological function during ageing in healthy individuals is imperative for comparison of future studies on diseased populations or disease pathology and progression.

6.6 Future research direction

The studies conducted in this thesis addressed the relationships of FGF21 and lung microbiome in ageing and exercise among healthy individuals. The results supported the potentially

important involvement of FGF21 and the lung microbiome in age- and exercise-associated changes in physiology and health. However, more research is needed to further the understanding on the downstream mechanisms of FGF21 on age-associated chronic diseases. For example, other downstream effectors involved in PPAR- α - and PPAR- γ -related pathways could be potential candidates for mediating the FGF21-associated improvement in glucose regulation or arterial stiffness. Since increased oxidative stress is a central mechanism driving age-related bone loss, other downstream mechanisms associated with the anti-oxidative pathway of FGF21 may be involved. Future research should also investigate whether the increase in circulating FGF21 concentrations with ageing and in sedentary individuals represent an increase in FGF21 resistance. Future studies may have the potential to translate FGF21 and lung microbiome into practical and effective therapeutic targets against age-associated chronic diseases. The future direction for research on this topic may include acute or chronic exercise interventions or longitudinal studies that provide greater insights of the mechanistic roles of FGF21 in ageing and exercise.

The studies in this thesis suggest the involvement of FGF21 in the age- and exercise-associated effects on glucose regulation, arterial stiffness and bone loss. However, these studies were done using a cross-sectional design and while associations can be drawn, they do not prove causality in inferring that FGF21 and its downstream mechanisms drive the various physiological changes with ageing and exercise. The current studies have shown the association between exercise habits and metabolic health, but the cross-sectional study design did not deeper the analysis on the causality between exercise habits and metabolic health outcomes. It would be useful to extend the existing studies by exposing the sedentary group to medium-term exercise programmes and to compare the metabolic response with the active group during and after the exercise intervention. Meta-analyses of larger cohorts and longitudinal or follow-up studies are

required to delineate these short-term and long-term effects of biological ageing and exercise on FGF21 and age-associated chronic diseases. While adiponectin and IGFBP1 may not be associated with FGF21, glucose regulation, arterial stiffness and bone loss in healthy individuals, longitudinal or intervention studies should be conducted in future to understand better the effects of ageing and exercise on FGF21, adiponectin and IGFBP, in mediating various physiological functions. Empirical investigations involving the administration of recombinant FGF21 with varying combinations of adiponectin or IGFBP1 in animals would be required to further understand the kinetics of FGF21 in mediating habitual exercise- and age-associated changes in physiology. Although the circulating concentrations of FGF21, adiponectin and IGFBP1 have been quantified in this study, the expression of adiponectin and IGFBP1 receptors were not accounted for and should be investigated in future studies in order to better delineate the role of adiponectin and IGFBP1 as downstream effectors of FGF21. It is possible that other downstream signaling mechanisms of FGF21 are involved in its associations with exercise and ageing health, or that FGF21 could act independently through direct signaling to organs, such as the brain, or through inter-organ crosstalk between the liver, adipose tissues and brain (307). For example, intra-cerebroventricular FGF21 injection resulted in higher energy expenditure and browning of adipose tissues in mice through direct binding to FGF21 receptor complex in multiple mice brain regions (366). Future research might investigate the FGF21 downstream signalling organs and associated mechanisms, on glucose regulation, arterial stiffness and bone loss in humans during ageing or with exercise.

A key limitation in the thesis was that the potential influence of lifestyle factors, such as diet, stress levels, and sleep habits on glucose regulation, arterial stiffness, bone health, FGF21 and the lung microbiome, were not accounted for. For example, 5-day dietary intervention in humans reported a 6-fold increase in circulating FGF21 concentration with 10% dietary protein

diet compared to a 25% protein diet (498). Consumption of high CHO diet (~80%) for 3 days in humans also led to an 8-fold increase in plasma FGF21 concentration, while a high fat diet (~78%) did not (499). In a systematic review of 28 randomized controlled trials including 6372 patients, lifestyle and diet modifications also improved metabolic syndrome risk factors after 1 year (500). Increased psychosocial and socioeconomic stress were associated positively with development of metabolic diseases (501). In a cross-sectional multi-ethnic study ($n = 2,003$), every 1 h increase in variability of sleep duration and timing were associated with 23–27% higher odds of metabolic syndrome (502). These studies collectively suggest that lifestyle factors can affect concentrations of biomarkers such as FGF21, and age-associated metabolic diseases, such as T2DM and cardiovascular diseases. Future studies involving a larger cohort should account for the effects of lifestyle-associated factors, such as diet, stress levels and sleep duration or variability on the parameters measured.

While this thesis examined the associated effects of habitual aerobic exercise on FGF21, in the context of glucose regulation and arterial stiffness, the effects of resistance exercise on FGF21 in age-associated chronic diseases are not known and warrant further investigations. Nonetheless, studies investigating the chronic and acute effects of resistance exercise on FGF21 reported an increase in FGF21 concentrations regardless of sex and age (503, 504). In older women (~56 years old), an 8-week circuit resistance training of 3 sessions/week increased FGF21 concentrations by 11% (503). An acute bout of resistance exercise training of 4 sets (8–10 repetitions each) at 70–75% of one repetition maximum, also increased post-exercise FGF21 concentration in young males (~23 years old) by ~2 fold (504). In that same study, the increase in FGF21 concentration post-exercise was significantly higher with resistance exercise than with high intensity interval training exercise, suggesting that exercise types may differentially affect FGF21 concentration and potentially influence age-associated diseases.

The current microbiome study is the first to investigate age-associated changes in the lung microbiome composition. Although the lung microbiome was associated with lung function and arterial stiffness in an age-dependent manner, the present study was unable to prove causality. Research evidence on the mechanisms that drive the specific microbial taxa changes that occur with ageing or age-associated decline in lung function and arterial stiffness are lacking currently. It is possible that age-associated alterations in immune function may affect the microbiome and generate low-grade systemic inflammation, which is a key contributor to respiratory and cardiovascular diseases (466). With ageing, microbial imbalance coupled with dysregulated host immune and inflammatory response may also lead to dysbiosis, affecting specific lung microbial composition that is related to lung function or arterial stiffening. Future studies should investigate the role of inflammation in mitigating the age-related associations between the respiratory microbiome and physiological functions. Longitudinal studies with a larger cohort would also be imperative to understand better the temporal dynamics of the lung microbiome during ageing. Elucidating the mechanisms underpinning FGF21 and the lung microbiome on age- and exercise-associated physiological changes, offer immense therapeutic and preventive potential for chronic diseases.

6.7 Conclusion

In conclusion, this thesis showed in healthy individuals that FGF21 but not adiponectin is associated with age- and habitual exercise-related effects on glucose regulation. The anti-oxidative function of FGF21 or adiponectin is not evident in age-associated increase in oxidative stress and arterial stiffness, among healthy individuals. However, FGF21 and adiponectin are independently and positively associated with arterial stiffness in sedentary but not in active individuals. FGF21 but not IGFBP1 is related to the age-associated increase in

bone turnover and decline in BMD. Age-associated changes in the specific lung microbiome taxa are also independently associated with changes in lung function and arterial stiffness. Taken together, these results suggest that FGF21 is associated with age-related decline in glucose regulation and bone mass and with the multipotent effects of exercise on arterial stiffness and glucose regulation. The lung microbiome may be a potential mechanism in the age-associated decline in lung function and arterial stiffness. The results imply that FGF21 and the lung microbiome are important factors that are associated with normative age-related physiological changes in healthy individuals, which support their possibility as effective targets to prevent or decelerate age-associated disease onset. FGF21 and the lung microbiome may also act as early markers or therapeutic targets for age-associated chronic diseases, which may potentially reduce the burden on healthcare systems and the economy.

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