Tampere University

BINISHA HAMAL MISHRA

Multiomic Biomarkers Associated with Early Traits of Atherosclerosis and Osteoporosis

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ACADEMIC DISSERTATION
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Responsible supervisor and Custos	Professor Terho Lehtimäki Tampere University Finland	
Pre-examiners	Docent Om Prakash Dwivedi University of Helsinki Finland	Adjunct professor Leo Niskanen University of Eastern Finland Finland
Opponent	Professor Laura Elo University of Turku Finland	

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Binisha Hamal Mishra

ABSTRACT

Atherosclerosis and osteoporosis are complex multifactorial diseases, each contributing to a significant disease burden and related health costs globally. Several studies have shown that these diseases share risk factors and pathophysiological mechanisms suggesting that they are co/multimorbid conditions. Follow-up studies of the diseases for in-depth understanding of common underlying molecular mechanisms are essential for joint prevention and therapeutics of these diseases. However, despite the strong evidence for co/multimorbidity hypothesis of the diseases, studies investigating the underlying molecular mechanisms of the diseases using omics data, such as transcriptomics and lipidomics, are lacking.

The aims of the study were, study I: Identification of lipidome-wide molecular lipid co-expression modules jointly associated with early markers of atherosclerosis and osteoporosis, study II: identification of transcriptome-wide gene co-expression modules jointly associated with early markers of atherosclerosis and osteoporosis, study III: lipidome-wide multivariate association analysis of early markers of both the diseases to identify molecular lipid species with potentially distinct biological role in the comorbidity/multimorbidity, study IV: identification of the shared biological processes underlying atherosclerosis-osteoporosis co/multimorbidity.

All the studies (I-IV) in this thesis are based on the data collected from ongoing Cardiovascular Risk in Young Finns Study (YFS). The YFS is a prospective multicenter follow-up study initiated in 1980 with 3596 children and adolescents aged 3 to 18 years randomly selected from the areas of five university hospitals in Finland, investigating cardiovascular risk factors from childhood to adulthood. The participants have been since followed up for over 40 years with regular intervals. Early markers of atherosclerosis used in this study included bulbus and carotid intima media thickness (CIMT) measured with high-resolution ultrasound during 2007 follow-up. Similarly, early markers of osteoporosis included indices of bone mineral density (BMD) and content, measured using peripheral quantitative computer tomography from the distal and shaft sites of tibia and radius during YFS 2007 follow-up at age 30-45 years.

In studies I and II, weighted co-expression network analyses of these early markers of atherosclerosis and osteoporosis were performed using lipidomic data profiled during 2007 using liquid chromatography-tandem mass spectrometry (study I) and genome-wide transcriptomic data profiled during 2011 from whole blood of YFS participants using Illumina HumanHT-12 version 4 Expression BeadChip (study II). The network analyses were performed to identify densely interconnected networks or modules of lipid species or genes that are associated with early markers of both the diseases. Joint association of the identified lipid or gene modules with the early markers of both diseases was assessed by performing multivariate analysis of variance (MANOVA). In study III, we performed MANOVA of the lipidome data to identify joint associations between each of the 437 individual molecular plasma lipid species and selected early markers of both diseases. In study IV, we performed gene set analysis (GSA) of the transcriptomic data to identify biological processes shared by the early markers of these two diseases. The GSA was done in case-control setting. Participants with high CIMT (>90th percentile) were defined as cases for subclinical atherosclerosis. In case of osteoporosis, study population-based T-scores for BMD were calculated and T-score ≤ -1 was used for the definition of low BMD cases.

In study I, we identified one plasma lipidomic module containing 105 lipid species that was significantly and jointly associated with early markers of both atherosclerosis and osteoporosis. Majority of lipid species in the module belonged to classes of glycerolipid (n=60), glycerophospholipid (n=13) and sphingolipid (n=29). Twenty of the lipid species belonging to class sphingolipid were plasma ceramides. In study II, we identified two gene modules significantly associated with the early markers of both diseases. The three most significant genes in the two identified gene modules were *NOSIP, GXYLT2* and *TRIM*. In study III, we identified four lipid species significantly associated with the early markers of both diseases. The four lipid species and the classes they belong to are TAG(18:0/18:0/18:1) (glycerolipid), PC(40:3) (glycerophospholipid), Gb3(d18:1/22:0) (sphingolipid) and Gb3(d18:1/24:0) (sphingolipid). In study IV, we did not identify any biological processes jointly associated with the early markers of both diseases. However, we identified three novel biological processes associated with high CIMT and replicated 234 gene sets significantly associated with high CIMT with false discovery rate (FDR) ≤ 0.01. These three novel biological processes were copper homeostasis, neural crest cell migration and nicotinate and nicotinamide metabolism.

The results from studies I-III support the atherosclerosis-osteoporosis co/multimorbidity hypothesis. They reveal a set of joint lipidomic and transcriptomic biomarkers for early signs of both the diseases which might imply their important role in developing dual-purpose prevention and/or treatment methods. The results of study IV show three gene sets representing three novel biological processes associated high CIMT, which might explain the transcriptomic link between these biological processes and atherosclerosis and serve as biomarkers of atherosclerosis. Methodologically, these studies (I-IV) highlight the importance of bioinformatic approaches such as weighted co-expression network analysis and gene set analysis for exploratory investigation of omics based shared structures and molecular features for co/multimorbidities.

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ABBREVIATIONS

APOE	apolipoprotein *E*
BIMTavg	average bulbus intima-media thickness
BIMTmax	maximum bulbus intima-media thickness
BMD	bone mineral density
BMI	body mass index
CIMTavg	average carotid intima-media thickness
CIMTmax	maximum carotid intima-media thickness
CoD	cortical density
CRP	C-reactive proteins
CVDs	cardiovascular diseases
DGE	differential gene expression
DO	disease ontology
DRToBMC	distal radius total bone mineral content
DRTrD	distal radius trabecular bone mineral density
DTToBMC	distal tibia total bone mineral content
DTTrD	distal tibia trabecular bone mineral density
FDR	false discovery rate
GO	gene ontology
GS	gene significance
GXYLT2	glucoside xylosyltransferase 2
HDL	high-density lipoprotein
IL-6	interleukin-6
IMT	intima-media thickness
KEGG	Kyoto encyclopaedia of genes and genomes
LDL	low-density lipoprotein
LPC	lysophosphatidylcholine
LRP	low-density lipoprotein receptor related protein
LS	lipid significance
MANOVA	multivariate analysis of variance
ME	module eigengenes or module eigenlipids

MET	metabolic equivalent hours
MM	module membership
MSigDB	molecular signatures database
NOSIP	nitric oxide synthase interacting protein
OPG	osteoprotegerin
P_{adj}	Bonferroni-adjusted p-value
pQCT	peripheral quantitative computed tomography
RANK	receptor activator of nuclear factor kappa-B
RANKL	receptor activator of nuclear factor kappa-B ligand
ROAST	rotation gene set test
RSCoBMC	radial shaft cortical bone mineral content
TNF-α	tumour necrosis factor-α
ToA	total area
TOM	topological overlap matrix
TrD	trabecular density
TRIM63	tripartite motif containing 63
TSCoBMC	tibia shaft cortical bone mineral content
YFS	The Cardiovascular Risk in Young Finns study

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text with their Roman numerals (I-IV):

Publication I. Mishra, B. H., Mishra, P. P., Mononen, N., Hilvo, M., Sievänen, H., Juonala, M., Laaksonen, M., Hutri-Kähönen, N., Viikari, J., Kähönen, M., Raitakari, O. T., Laaksonen, R., & Lehtimäki, T. (2020). Lipidomic architecture shared by subclinical markers of osteoporosis and atherosclerosis: The Cardiovascular Risk in Young Finns Study. *Bone*, *131*, 115160. https://doi.org/10.1016/j.bone.2019.115160

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of osteoporosis: Atherosclerosis-osteoporosis co/multimorbidity study in the Young Finns Study. *Atherosclerosis*, *361*, 1–9. https://doi.org/10.1016/j.atherosclerosis.2022.10.005

Author's contribution

Publications I: The author contributed to the overall conceptualization of the study, to define the research question and to the planning of the bioinformatics analysis methodology to identify network of lipid species associated with early markers of osteoporosis and atherosclerosis. The author performed all the biostatical analysis, including weighted co-expression network analysis and multivariate analysis of variance used in the study. The author interpreted the result, wrote the first draft of the manuscript, and took full responsibility in revising the manuscript addressing the external reviewers up to final publication.

Publication II. The author adapted the bioinformatic analysis pipeline developed in study I to identify transcriptomic architecture shared by early markers of osteoporosis and atherosclerosis. The author performed all the biostatical analysis, including weighted co- expression network analysis, multivariate analysis of variance and pathway analysis used in the study. The author interpreted the result, wrote the first draft of the manuscript, and took full responsibility in revising the manuscript addressing the external reviewers up to final publication.

Publication III: The author conducted multivariate analysis of variance of the human serum lipidome data to identify molecular lipids that are jointly associated with early markers of osteoporosis and atherosclerosis. The author interpreted the result, wrote the first drafts of the manuscripts, and took full responsibility in revising the manuscript addressing the external reviewers up to final publication.

Publication IV: The author conducted gene set analysis of the transcriptome data to identify biological process jointly shared by early markers of osteoporosis and atherosclerosis. The author interpreted the result, wrote the first drafts of the manuscripts, and took responsibility in revising the manuscript addressing the external reviewers up to final publication.

1 INTRODUCTION

Atherosclerosis and osteoporosis are both widely prevalent disorders, contributing to significant disease burden worldwide with serious morbidities and death (Berenson et al., 1998; Cummings & Melton, 2002; Hernlund et al., 2013; Jørgensen et al., 2004). It is estimated that roughly 17.9 million people died from cardiovascular disease (CVD) in 2019, which accounts for 32% of all deaths globally (Libby, 2021; Roth et al., 2020). The estimated number of fragility fractures in the European countries in 2010 was 3.5 million, and it is predicted to rise by 28%, reaching 4.5 million by 2025, which imposes a devastating burden on sufferers, their families and health care system (Kanis et al., 2019). Studies indicate that atherosclerosis and osteoporosis are co/multimorbid conditions sharing common risk factors, pathophysiological mechanisms, and genetic factors (Farhat & Cauley, 2008; Hamerman, 2005; Szekanecz et al., 2019). Both diseases often progress symptomless without major clinical event such as myocardial infarction due to atherosclerosis or bone fracture due to osteoporosis.

Multimorbidity attributes high demands on health care system around the world (Marengoni et al., 2011). Comorbidity is when one or more disease is present during the clinical course of patient in relation to the primary disease of interest(Feinstein, 1970). However, multimorbidity is when there is co-existence of two or more chronic conditions, where no one condition is identified as primary condition(Boyd & Fortin, 2010). The global prevalence of co/multimorbidity has increased and is expected to continue to rise (MacMahon & The Academy of Medical Sciences, 2018). For example, it is estimated that 50 million people suffer from co/multimorbidity in the European Union alone (Jansen et al., 2014). Although, co/multimorbidy is particularly common in elderly, it is not limited to elderlies. Co/multimorbidy is reported in 79% of people with some sort of disease (Mezzich & Salloum, 2008), of those with co/multimorbidity, 42.1% were younger than 60 years of age (Taylor et al., 2010). Therefore, there is an urgent need for deeper understanding of the co-existence of various disease, identify common risk factors,

joint molecular mechanism, and develop common methods for risk stratification, prevention, diagnosis, and treatment.

There have been several omics studies investigating biomarkers responsible for pathogenesis of atherosclerosis and osteoporosis independently. However, omics studies investigating both osteoporosis and atherosclerosis phenotypes combined are still lacking. Therefore, in this study we used lipidomic and transcriptomic data to investigate joint association of lipids/genes with early markers of atherosclerosis and osteoporosis.

Lipidomics offers a tool to investigate the cellular metabolism by quantifying the changes of individual lipid classes, subclasses and molecular species that reflect metabolic differences(K. Yang & Han, 2016). Understanding these changes can provide useful insight into the development process of diseases. Furthermore, transcriptomics offers the opportunity to investigate gene structure, expression, regulation, and functional implications of the genetic variability (Casamassimi et al., 2017; B. Wang et al., 2019).

Therefore, the goal of this study was to perform system as well as molecular-level analysis of lipidomics and transcriptomic data to identify lipid species or genes and their networks associated jointly with early markers of atherosclerosis and osteoporosis.

2 LITERATURE REVIEW

2.1 Osteoporosis

Osteoporosis is a bone disease characterized by low bone density and porous bone leading to a decrease in bone strength, making them susceptible to bone fracture (Christodoulou & Cooper, 2003). Osteoporosis is known as a silent disease as we cannot see or feel bone loss, and it goes unnoticed until it becomes so weak that a sudden strain, bump, or fall can cause a fracture (Kanis et al., 2019; Liscum, 1992). Osteoporosis causes more than 9 million bone fractures across both men and women every year resulting in an osteoporotic bone fracture every 3 second (Johnell & Kanis, 2006). Globally, 1 in 3 women and 1 in 5 men over the age of 50 will experience osteoporotic fracture. After the first fracture, these women are 5 times more likely to suffer another bone break within a year (Kanis et al., 2019). In Europe, fragility fractures will rise from 3.5 million in 2010 to 4.5 million in 2025 which corresponds to an increase of twenty eight percent (Kanis et al., 2019). Osteoporosis and osteoporotic fractures cause large numbers of disabilities, deaths, and huge health care costs through hospital and rehabilitation expenses (Kanis et al., 2019).

2.2 Bone structure

Bone is a highly specialized form of connective tissue that makes up approximately 10-15% of the total body weight (Mizokami et al., 2017). Bone provides shape and support for the body, protects internal organs, and provides storage for minerals like calcium and phosphorous that can be released to the body as needed. Bone also provides a medium for bone marrow for the development and storage of blood cells (Su et al., 2019). Bone is made up of two types of bone tissues,

cortical and trabecular (Adler, 2000). The detailed bone structure is shown in Figure 1.

2.2.1 Cortical bone

Cortical bone, also known as compact bone, is dense and compact external layer of bone which makes around 80% of the adult bone mass (Hadjidakis & Androulakis, 2006). It gives bone its smooth and white appearance. Compact bone consists of osteon or haversian system, which is the morphological and functional unit of cortical bone [Figure 1] (Clarke, 2008). The osteon consists of central canal called the osteonic or haversian canal which is surrounded by concentric rings of matrix called lamellae. These haversian canals contains nerve fibers, lymphatic vessels, and blood vessels. Between the lamellae, the bone cells, osteocytes, are in oblong spaces called lacunae. Small channels (canaliculi) radiate from the lacunae to provide passageways for nutrients. Adjacent haversian or osteonic canals are connected via a canal called Volkmann's canal. Cortical bone facilitates bone's main functions to provides mechanical and protective functions (Clarke, 2008).

2.2.2 Trabecular bone

Trabecular bone, also called as cancellous or spongy bone is light, flexible, porous, and are less dense than cortical bone and accounts for 20% of the total bone mass [Figure 1] (Hadjidakis & Androulakis, 2006). Trabecular bone exists at the end of long bones and in the middle of short, flat, and irregular bones. Trabecular bone is organized into a network of interconnected rods and plates called trabeculae which are its anatomical and functional units (Clarke, 2008). The space between the trabeculae helps the bone to be lighter and easier to mobilize. There are openings on the trabeculae called canaliculi which connects adjacent spaces for blood supply. Trabecular bone is highly vascular and contains bone marrow within these cancellous bone spaces where hematopoiesis occurs. Hematopoiesis is the formation of blood cells including platelets, red blood cells and white blood cells from hematopoietic stem cells in the bone marrow (Clarke, 2008).

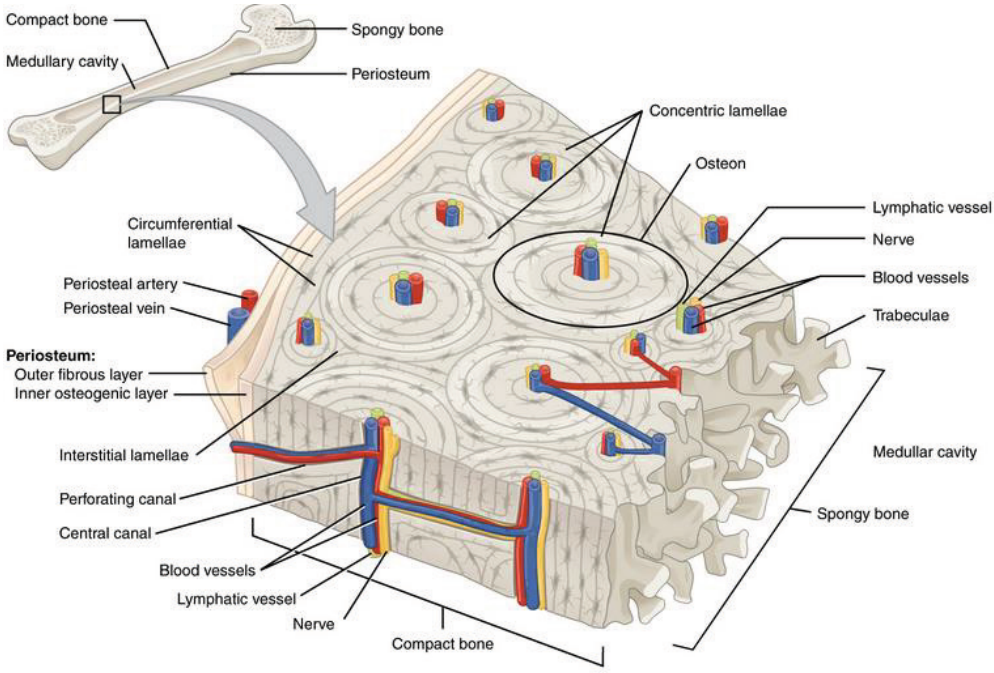

Figure 1. Bone Structure. Source: Structure and components of bone Anatomy & Physiology, Connexions Web site. http://cnx.org/content/col11496/1.6

2.3 Bone composition

Bone is composed of two main compartments, bone matrix and bone cells forming the bone matrix.

2.3.1 Bone matrix composition

Bone matrix comprises of organic and inorganic components (Feng, 2009) that allows the bones to be strong and rigid, but not too brittle [Figure 2].

2.3.1.1 Organic components

Organic component, also known as osteoid, consists of type I collagen (~90%) and non-collagenous proteins (Hadjidakis & Androulakis, 2006). Type I collagen is the most abundant form of intrinsic collagen found in the bone that is secreted by osteoblasts. Non-collagenous organic materials are endogenous proteins such as glycoprotein, osteocalcin, and proteoglycans. The orientation of the highly organized collagen fiber along with other protein helps the bone to give its flexibility and tensile strength (Hadjidakis & Androulakis, 2006).

2.3.1.2 Inorganic components

Inorganic components of bone comprise of mineral salts such as calcium and phosphorous that form hydroxyapatite [Figure 2]. Hydroxyapatite incorporates with the other inorganic salts like magnesium hydroxide, fluoride, and sulfates as it calcifies on the collagen fiber (Feng, 2009). The hydroxyapatite crystal gives bones its hardness and strength.

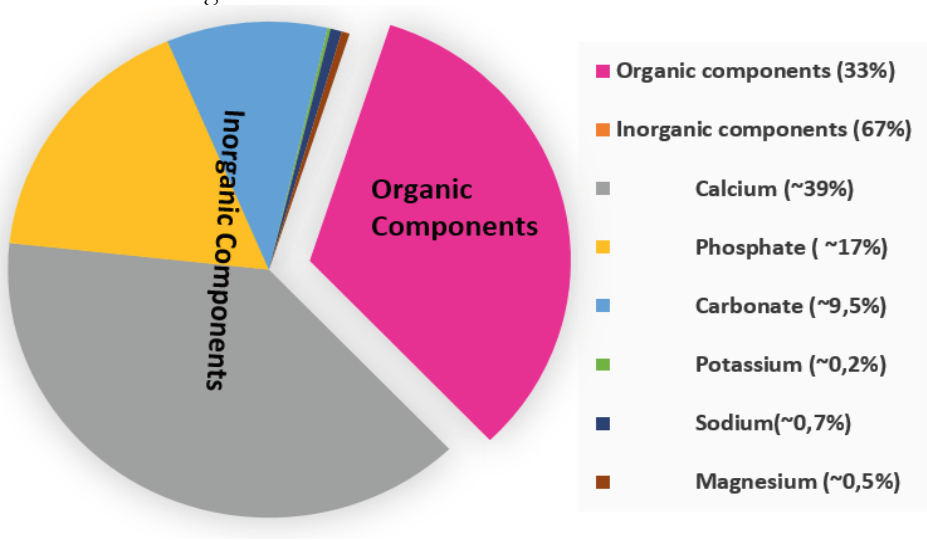

Figure 2. Composition of bone

2.3.2 Bone cells

Bone is composed of four different cell types: osteogenic (osteoprogenitor) cells, osteoblast, osteocyte, and osteoclast.

2.3.2.1 Osteogenic (osteoprogenitor) cells

Osteogenic cells are derived from mesenchymal cells. Mesenchymal cells are the embryonic tissue from which almost all the connective tissues are formed (Nahian & Davis, 2020). Osteogenic cells are the only bone cells that undergo cell division. These cells differentiate and develop into osteoblasts. In matured bone surfaces that are neither in formative nor resorptive phase, osteoprogenitor cells line the bone surface and exist as flattened elongated cells termed as bone-lining cells (Mohamed, 2008).

2.3.2.2 Osteoblast

Osteoblasts are cells responsible for building bones. These cells are responsible for synthesizing and secreting collagen fibers and other organic components that make up the bone matrix (Clarke, 2008). Osteoblasts are also responsible for producing alkaline phosphatase, which is the enzyme responsible for forming hydroxyapatite, a mineral portion of the bone (Hadjidakis & Androulakis, 2006). Once osteoblast has synthesized enough protein, collagen fiber, and alkaline phosphatase to form the organic and inorganic portion of the bone matrix around them, the osteoblast calcifies, and become trapped in their own secretions (Capulli et al., 2014). Once this happens, the osteoblast differentiates into osteocytes.

2.3.2.3 Osteocyte

Osteocytes are mature version of osteoblast within the bone matrix and make up 90–95% of the cellular content of bone. They occupy the spaces within the bone matrix called lacunae (Hirose et al., 2007) and network with each other via a long cytoplasmic extension which are little arm or branches, called canaliculi. Unlike osteoblast and osteoclast cells, osteocytes can communicate with around 50 neighboring osteocytes (Creecy et al., 2021). Therefore, any stimulus triggered on osteocytes have greater impact in bone turnover, either positively or negatively. Furthermore, osteocytes play important role in mechanotransduction, a mechanism by which cells convert mechanical stimuli to cellular responses, for maintaining well-balanced bone homeostasis (Choi et al., 2022). The energy and macromolecule requirement of this process is fulfilled by metabolism of nutrients (Romani et al., 2021). This crosstalk between mechanotransduction and metabolism might reflect as links between metabolomics and bone density.

2.3.2.4 Osteoclast

Osteoclasts are derived from the macrophage-monocyte cell lineage rather than from osteogenic cells (Väänänen et al., 2000). These cells are developed from the fusion of monocytes in the bone marrow resulting in large multinucleated cells (Boyle et al., 2003). Osteoclast has distinctive structure due to deep folding of the plasma membrane in the area facing the bone matrix (called ruffled border) and the surrounding zone of attachment (called sealing zone) (Hadjidakis & Androulakis, 2006). The ruffled border of osteoclast secretes lysosomal enzymes and acids such as tartrate-resistant acid phosphatase and cathepsin K that breaks down and digest extra cellular matrix components. This process of breakdown of bone is called bone resorption (Hadjidakis & Androulakis, 2006). During the breakdown of extracellular matrix, there is release of minerals such as calcium and phosphate into blood which plays role in regulating blood calcium level (Kenkre & Bassett, 2018; Väänänen et al., 2000).

Osteoclasts continually break down old bone cells while osteoblasts continually form new bone cells, whereby old bone tissue is replaced by new bone tissue. The balance

between osteoblasts and osteoclasts is responsible for maintaining bone strength and mineral homeostasis (Damsky, 1999; J. M. Kim et al., 2020).

2.4 Bone Remodeling

Bone remodeling is the process when old brittle bone tissue is removed or resorbed and gets replaced by new bone tissue. This process continues throughout life and most of the adult skeleton is replaced about every 10 years. Spongy bone is replaced every 3 to 4 years and compact bone is replaced every 10 years. The remodeling process takes place in five steps as listed below [Figure 3], occurring over the course of 4-8 months throughout life (Kenkre & Bassett, 2018).

2.4.1 Activation

Osteoclast precursor cells are recruited from the circulation and activated through the retraction of the bone lining cells and the digestion of the endosteal membrane by collagenase action (Raggatt & Partridge, 2010). Once bone surface is exposed, the lining cells separate from underlying bone and these pre-osteoclasts become fused and form multinucleated osteoclasts.

2.4.2 Resorption

Osteoclasts begin to dissolve the mineral matrix and decompose the osteoid matrix, leaving holes or cavities in focal areas of the bone called as resorption pit (Sims & Martin, 2014). During this process, the osteoclast releases calcium and phosphate into the bloodstream. The resorption phase is terminated by osteoclasts programmed cell death, ensuring that excess resorption does not occur (Kenkre & Bassett, 2018).

2.4.3 Reversal

In the reversal phase, osteoblastic lineage cells remove unmineralized collagen matrix from the resorption pit and mesenchymal stem cells are attracted along the resorption pit. The cells along the pit proliferate and differentiate to become into pre-osteoblasts cells (Sims & Martin, 2014).

2.4.4 Formation

During the formation phase, the pre-osteoblasts mature into osteoblasts at the surface of the resorption pit. The osteoblasts begin to build bone by synthesizing and secreting type 1 collagen-rich osteoid matrix, which is a soft, nonmineralized bone matrix. Osteoblast also absorb calcium and phosphate from blood to create hydroxyapatite crystal and are deposited amongst collagen fibrils, thus creating a new bone (Atkins & Findlay, 2012; Kenkre & Bassett, 2018).

2.4.5 Termination

Once mineralization is complete, osteoblasts change into flattened bone-lining cells and remain dormant until a new remodeling cycle is initiated (Kenkre & Bassett, 2018; Raggatt & Partridge, 2010).

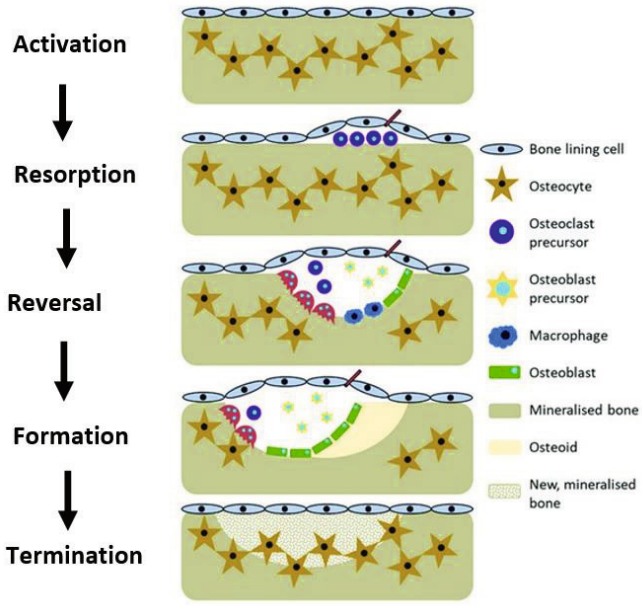

Figure 3. The five-stage bone remodeling cycle. Bone turnover follows a sequence of events that includes activation, resorption, reversal, formation, and termination. Modified from Owen, R. & Reilly, G. C. In vitro models of bone remodeling and associated disorders. Frontiers in Bioengineering and Biotechnology 6, (2018).

2.5 Pathophysiology of osteoporosis

In a normal bone remodeling process, balance between bone resorption and formation is maintained to ensure that the overall bone volume and structure remain unchanged. However, an excessive bone remodeling and accelerated bone loss may occur under certain pathological conditions, such as osteoporosis (Kanis et al., 2019; Raisz, 2005). Bone resorption is initiated through production of RANKL (Receptor activator of nuclear factor kappa-B ligand) by osteoblast cells. Osteoblast also expresses and releases Osteoprotegerin (OPG), that mimic the RANK (Receptor activator of nuclear factor kappa-B) receptor, which is a natural inhibitor of RANKL and plays in role in bone resorption (Kong et al., 1999). During the activation phase of bone remodeling, bone lining cells move apart to expose the bone surface (Raggatt & Partridge, 2010). Osteoblast then begins expressing RANKL. RANKL binds to RANK on osteoclast precursors cells. This initiates cell fusion and formation of

mature multinucleated osteoclast. RANKL continues to bind to RANK on mature osteoclasts. The binding of RANKL to RANK is essential for osteoclast formation, function, and survival [Figure 4] (Bolamperti et al., 2022; Raisz, 2005).

The process of bone remodeling is regulated by factors like OPG and estrogen (Bolamperti et al., 2022; Srivastava et al., 2001). OPG blocks the binding of RANKL to RANK thereby reducing the osteoclast activity. Estrogen limits the amount of RANKL expression by osteoblast. Reduced level of estrogen led to increased expression of RANKL by osteoblasts. Excessive RANKL overwhelm OPG leading to more osteoclast cells, increase in bone remodeling activity and greater bone loss. Bone resorption occurs at greater rate than bone formation and therefore, resorption pit cannot be completely refilled which overtime leads to thinning and weakening of bone. The progressive loss of bone following osteoporosis reduces the structural integrity and strength of the skeleton, thus leading to osteoporosis (Raisz, 2005; Raisz & Prestwood, 2000).

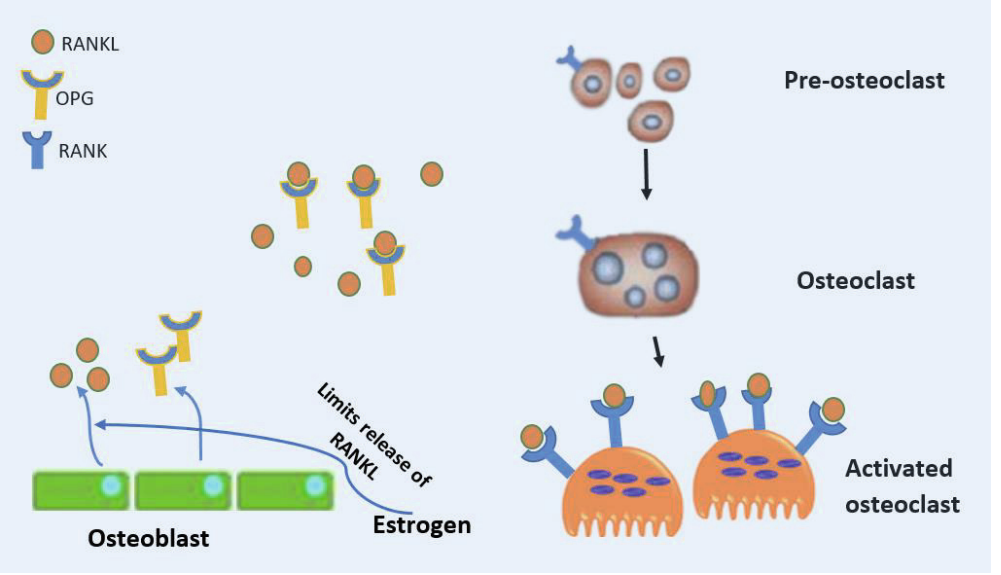

Figure 4. RANKL-RANK-OPG interplay in bone remodeling. Osteoblasts produce RANKL (Receptor activator of nuclear factor kappa-B Ligand) and osteoprotegerin (OPG) under the control of

various cytokines, hormones, and growth factors. OPG binds and inactivates RANKL, resulting in the inhibition of osteoclastogenesis. Estrogen limits the amount of RANKL expression by osteoblast. RANKL binds to RANK (Receptor activator of nuclear factor kappa-B) enhancing osteoclastogenesis. When RANK binds to RANKL receptor on the surface of pre-osteoclast, it triggers several intracellular processes differentiation into mature osteoclasts, thus activating bone resorption.

2.6 Atherosclerosis

Cardiovascular diseases (CVDs) are the leading cause of morbidity and mortality globally (Roth et al., 2020). CVD is a term that encompasses various conditions affecting heart and circulatory system. Atherosclerosis is one of the main contributors to CVD which begins decades before the clinical manifestations and silently reaches a stage where it can only be slowed down but not be reversed (Epstein et al., 1992; Libby, 2021). Atherosclerosis is derived from the Greek words 'athero' meaning gruel and 'scleros' meaning hard which was coined by Félix Marchand in the year 1940. Atherosclerosis is characterized by buildup of fatty substance, cholesterol, cellular waste product and calcium, called plaque or atheroma, on the innermost lining of the arteries (Lorkowski & Cullen, 2007). This process thickens the arteries causing complete or partial blockage of blood flow to organs and other tissue structure (Drexler, 1998a; Libby, 2021). Plaque formation in the coronary arteries lead to myocardial ischemia resulting in a heart attack (Singh et al., 2002). Atherosclerosis in carotid artery decreases or completely blocks the flow of blood to the brain leading to stroke (Hansson, 2005). Furthermore, peripheral vascular disease is caused by plaque formation in blood vessel other than heart and brain (Hansson, 2005; Libby, 2021; Lorkowski & Cullen, 2007; Singh et al., 2002).

2.6.1 Structure of normal artery

Arteries have three primary layers: tunica intima, tunica media, and tunica adventitia [Figure 5] (Tucker et al., 2021). Tunica intima is the innermost and thinnest

layer of the artery. It is lined with endothelial cells which are in direct contact with red blood cells in the lumen. Surrounding the tunica intima is the tunica media which is comprised of smooth muscle cells which not only provide support for the artery but also control the diameter of the artery by contracting or relaxing in response to blood flow and blood pressure. The outer most layer is tunica adventitia which attaches the artery to the surrounding tissue. This layer consists of connective tissues arranged circularly around the artery with varying amounts of elastic and collagenous fibers.

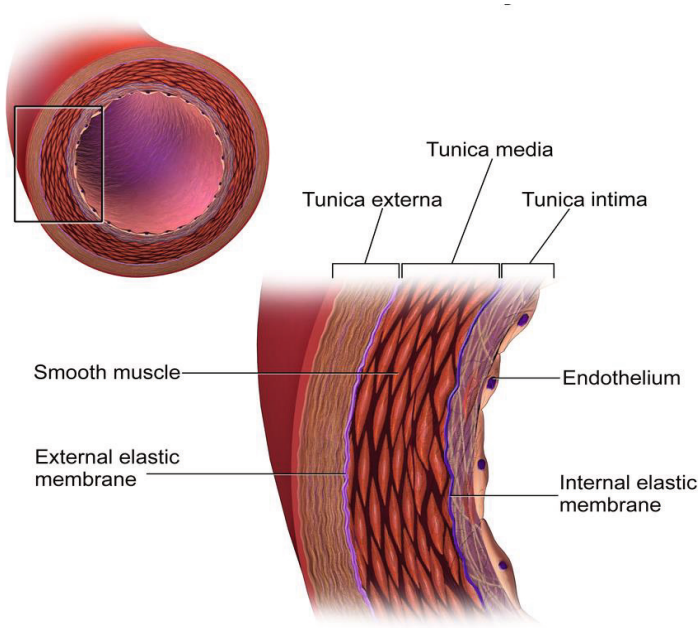

Figure 5. Overview of the structure of a normal arterial vessel. Blood vessel layers: Arteries consist of three layers: an outer tunica externa, a middle tunica media, and an inner tunica intima. Source: Blausen.com staff (2014). "Medical gallery of Blausen Medical 2014". *WikiJournal of Medicine* 1 (2). DOI:10.15347/wjm/2014.010. ISSN 2002-4436

2.6.2 Classification of atherosclerosis lesions

American Heart association provides classification of human atherosclerotic lesions based on their histological composition and structure (Stary et al., 1994, 1995). This classification includes six different categories [Table 1], namely Type I, Type II, Type III, Type IV, Type V and Type VI. Type V and VI are further subdivided into Va, Vb and Vc and VIa, VIb and VIc.

Table 1. Classification of human atherosclerotic lesions provided by American Heart association.

Nomenclature	Histological classification of atherosclerotic lesion
Type I Initial Lesion	Lesion consists of atherogenic lipoprotein, isolated groups of macrophages containing lipid droplets (macrophage foam cells)
Type II Fatty streak Lesion	Abundant macrophage foam cells interspersed within a smooth muscle cell.
Type III Intermediate Lesion	Lesions contain extracellular lipid droplets and particles, accumulation of macrophages on the luminal aspect of the plaque and layers of smooth muscle cells.
Type IV Atheroma Lesion	Lesion contains lipid core that is well-formed with foam cells and extracellular deposits and between the lipid core and the endothelial surface, the intima contains macrophages and smooth muscle cells with and without lipid droplet inclusions.
Type V	Several lipid cores, separated by thick layers of fibrous connective tissue, are stacked irregularly one above the other.
Type Va Fibroatheroma Lesion	
Type Vb Calcific Lesion	Lesions containing large amount of calcium deposits which replaces the accumulated remnants of dead cells and extracellular lipid, including entire lipid cores.
Type Vc Fibrotic Lesion	Often present in arteries of the lower extremities, lipid core is absent, the normal intima is replaced and thickened with fibrous connective tissue, while lipid is minimal or even absent.
Type VI Complicated Lesion	Hematoma or hemorrhage
Type VIa	

Type VIb	Thrombosis
Type VIc	Presence of all three, namely hematoma, hemorrhage, and thrombosis.

2.6.3 Pathophysiology of atherosclerosis

The progression of atherosclerosis begins when the endothelial cell is damaged [Figure 6] (Libby, 2021; Lüscher & Barton, 1997). This can be caused by conditions such as hypertension, smoking, hyperglycemia and hypercholesteremia (Russell, 1999). Endothelial damage increases the permeability of the arterial wall allowing LDL (low-density lipoprotein) to enter the tunica intima (Davignon & Ganz, 2004). When LDL is oxidized it cannot leave the tunica intima. Oxidized LDL activate endothelial cells causing the endothelial cells to express adhesion molecules for white blood cells (monocytes) on their surfaces (Tabas et al., 2007). This allows monocytes and T helper cells to move into the tunica intima layer of the blood vessel (Gisterå & Hansson, 2017). When monocytes move into tunica intima, they differentiate into macrophages and then these macrophages engulf oxidized LDLs. As macrophages engulf more and more oxidized LDL particles they are known as foam cells (Cagnina et al., 2022a; Steinberg & Witztum, 2002). Foam cells undergoes apoptosis and release its lipid content in the tunica intima. Eventually the accumulation of lipids from the described process and the fragments of dead cells forms an area with lipid core that begins to form a plaque (Cagnina et al., 2022b; Ip et al., 1990). Plaques keep growing overtime as it accumulates calcium salts and dead cells causing it to harden. Foam cells promote migration of smooth muscle cells from tunica media to tunica intima (Cagnina et al., 2022b; Drexler, 1998b). In the tunica intima the smooth muscle cell proliferates and secrete extracellular matrix substance like collagen which forms a protective cap over the plaque and thus prevents the plaque from rupturing. As atherosclerotic plaques advance, the blood vessel narrows, which could result in blockade of blood flow. Furthermore, as the plaque grows, it builds pressure causing rupture of the fibrous cap and an area of plaque may jet out into the blood vessel (Frink, 2002). This can trigger blood clot or thrombosis, consequently impeding blood flow that can cause serious complications.

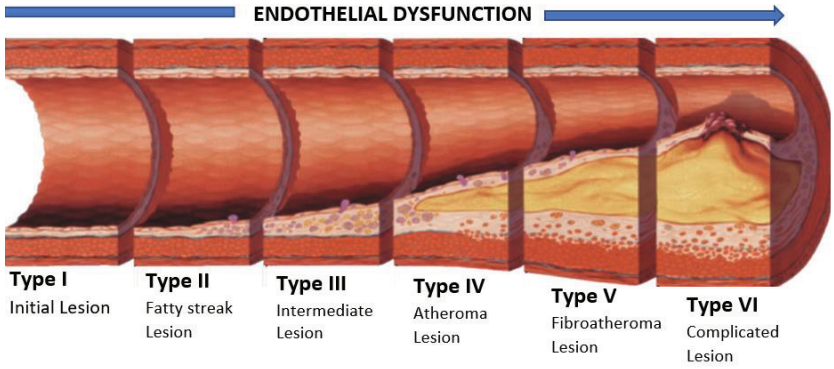

Figure 6. Illustration showing the mechanisms and stage of atherosclerotic plaque that led to initiation and progression of the atherosclerotic lesions. Modified from YitzhakNat, CC BY-SA 4.0 https://creativecommons.org/licenses/by-sa/4.0, via Wikimedia Common

2.7 Common risk factors and mechanism in the development of atherosclerosis and osteoporosis

Atherosclerosis and osteoporosis share common risk factors such as ageing, smoking habit, low physical activity, alcohol intake and hypertension. Furthermore, these diseases also share several common pathophysiological mechanisms involving estrogen, inflammatory cytokines, lipid oxidation products, vitamin D and K deficiency, as well as genetic biomarkers, such as OPG, and the matrix gla protein (Farhat & Cauley, 2008; Hamerman, 2005; Han, 2016; Szekanecz et al., 2019; Mo et al., 2022).

2.7.1 Aging

Ageing is a progressive degeneration of wide variety of molecular and cellular tissue overtime which gradually effects the structure and function of vital organs leading to gradual decrease in physical, mental, and social capacity. Aging is the predominant risk factor for most chronic diseases, atherosclerosis and osteoporosis

being few of them. Bone cells undergoes continuous removal of old bone cells by osteoclasts followed by the deposition of new bone cells by osteoblast which are coupled tightly to maintain bone mass and strength (Demontiero et al., 2012). Ageing causes significant shift of balance with greater bone resorption and less bone formation leading to gradual and progressive decline in bone mass, thus increasing risk for fragility fractures (Raisz & Rodan, 2003). Aging of vascular tissue results from exposure to various physical and metabolic stress and accumulation of metabolic by-products over time. This process can adversely promote endothelial dysfunction which increase monocyte/endothelial adherence, enhanced endothelial cell apoptosis and in turn, promote atherosclerosis (J. C. Wang & Bennett, 2012).

2.7.2 Vitamin K, matrix gla protein and osteocalcin

Vitamin K naturally exists in two main forms, vitamin K1 and vitamin K2. Vitamin K2, also known as also called "menaquinones, plays a pivotal role in the maintenance of bone health and vascular health through regulation of calcium homeostasis (Mandatori et al., 2021). Most vitamin K2 is produced in the intestine by intestinal bacteria, however, vitamin K2 derived from intestinal bacteria is poorly absorbed and hence not enough to maintain the normal physiological functions (Conly & Stein, 1992). Therefore, vitamin K2 should be supplemented via dietary food intake such as meat and egg yolk and from fermented dietary sources, such as curd cheese (Suttie, 1995). Meta-analysis of 19 clinical trials including 6759 participants showed the role of vitamin K2 in the maintenance and improvement of vertebral bone mineral density and the prevention of fractures in postmenopausal women with osteoporosis (Huang et al., 2015). Furthermore, in Rotterdam study, vitamin K2 was positively associated with coronary heart disease and inversely associated with all-cause mortality and severe aortic calcification supporting the cardioprotective effects of vitamin K2 intake (Geleijnse et al., 2004; Hariri et al., 2021).

Effect of vitamin K on bone demineralization and vascular calcification is mediated by gla-containing proteins such as matrix gla protein and osteocalcin. Vitamin K2 acts as coenzyme for glutamate carboxylase which converts glutamic acid to γ-carboxyglutamate (gla) protein (Furie et al., 1999; Wen et al., 2018). Matrix Gla proteins are thought to be a regulatory protein responsible for inhibiting

mineralization via its ability to bind to calcium crystals (Vassalle & Iervasi, 2014; Zoch et al., 2016). Higher expression of matrix gla protein increases bone mass via suppressing osteoclastogenesis and reduced matrix gla protein stimulates osteoclast differentiation and its function (Y. Zhang et al., 2019). Furthermore, mice that lack matrix Gla protein died from blood vessel rupture due to arterial calcification indicating its potent role in vascular calcification (El-Maadawy et al., 2003; Luo et al., 1997). Osteocalcin has been found in calcified atherosclerotic plaque lesions, and production of this protein is up regulated in people with atherosclerosis (Tacey et al., 2018; Vermeer et al., 2004). Osteocalcin knock-out in mouse showed increased bone loss after ovariectomy, indicating a protective role for osteocalcin (Vermeer et al., 2004). Hence, vitamin K2 might be a natural compound that may be able to prevent and/or treat metabolic bone and vascular disease such as osteoporosis and atherosclerosis.

2.7.3 Oxidized lipids

Lipids are a major component of food and important structural and functional constituents of cells in biological systems. Lipid function as long term energy source, thermal insulation, protection and as signaling molecules. Cholesterol and triglycerides are the major lipids in humans and are transported across body with lipoproteins. Lipoprotein is composed of central core consisting of cholesterol esters and triglycerides surrounded by an outer shell of phospholipids, free cholesterol and apolipoproteins. Substantial evidence indicates that oxidized lipids, lipids that have undergone oxidative modifications and lipoproteins are useful marker for both atherosclerosis and osteoporosis (Birukov, 2006; Parhami, 2003). Oxidized lipids induce human T lymphocytes to secrete RANKL, a key mediator of osteoclast differentiation and activity (Graham et al., 2009). Oxidized lipids are known to promote inflammation through generation of inflammatory factors such as cytokines that contribute to bone loss by increasing osteoclast activity and decreasing bone formation (Parhami, 2003). Likewise, oxidized lipoprotein particles alter osteoblastic cell proliferation, migration, and apoptosis through oxidative stress, and thus, affecting bone metabolism equilibrium (Hamel et al., 2008). Oxidized lipoprotein particles have also been detected in atherosclerotic lesions (Shen et al., 2020).

Oxidized lipoprotein especially LDL exhibits many atherogenic activities including endothelium dysfunction, monocyte differentiation, macrophage activation, foam cell formation, SMC migration and proliferation, platelet activation and aggregation (Khatana et al., 2020, Leiva et al., 2015). Hence, oxidized LDL play substantial role in the pathogenesis of atherosclerosis (Lehtimäki et al., 1999; Oksala et al., 2010) and osteoporosis.

2.7.4 Dyslipidemia

Dyslipidemia refers to imbalance of lipids, that may be manifested as elevation of total cholesterol, LDL cholesterol and triglyceride and a decrease in the high-density lipoprotein (HDL) cholesterol concentration. Dyslipidemia is the most important risk factor of cardiovascular diseases (Hedayatnia et al., 2020). High lipid levels, especially the elevated level of LDL cholesterol and total cholesterol have been shown to be strongly related to the accumulation of cholesterol within the arterial wall. Oxidative modification of LDL initiates inflammatory responses and promote uptake of macrophages and ultimately converting them to foam cells. Elevated level of triglyceride is associated with increased risk of cardiovascular disease (Nordestgaard & Varbo, 2014). Triglyceride rich lipoproteins migrate into the arterial wall and lead to an accumulation of cholesterol that promote plaque formation in atherosclerosis lesion (Talayero & Sacks, 2011). High level of total cholesterol and LDL cholesterol have been shown to associated with lower bone mineral density in postmenopausal women group (Adami et al., 2004; Jeong et al., 2014; X. L. Yang et al., 2019). Increased cholesterol level inhibits osteoblast differentiation and increases osteoclast activity preventing bone formation (Mandal, 2015). While there is no direct relationship between triglycerides and osteoporosis, studies have suggested that high triglyceride levels may be associated with a higher risk of bone loss and fractures (ANDO et al., 2016; X. L. Yang et al., 2019) . However, other study suggested that osteoporotic tissue demonstrated lower level of triglyceride as compared to normal healthy control (Dragojevič et al., 2013).

2.7.5 Vitamin D

Vitamin D is a major steroid hormone that comes in two major forms, vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol Vitamin D2 generally comes from plants, especially mushrooms and yeast, whereas vitamin D3 comes from animal sources, such as oily fish, liver, and eggs. (Durrant et al., 2022). Vitamin D regulate calcium and phosphorus absorption which is important to maintain adequate calcium concentrations required for the normal mineralization of the bone. Deficiency of vitamin D decreases calcium absorption in the intestine which results in mobilizing calcium and phosphorus from bones to maintain optimal level of calcium and phosphorus in the body leading to dysfunctional bone homeostasis (Stojanovic et al., 2011). Low level of vitamin D is associated with low bone mineral density which may lead to osteoporosis and related fractures (Lips & van Schoor, 2011). Vitamin D has protective role in maintaining endothelial integrity, a key component in the initiation of the atherogenic process (Kassi et al., 2013). Low vitamin D levels are associated with increased risk for development of atherosclerotic lesion via vascular smooth muscle cells proliferation, migration, and immune response modulation (Kassi et al., 2013). Hence, vitamin D deficiency may play an important role in the pathogenesis of both atherosclerosis and osteoporosis.

2.7.6 Estrogen

Estrogen is a class of steroid hormones that regulate growth, development, and physiology of the human reproductive system, especially female (Lee et al., 2012). While the estrogen levels are significantly lower in males, estrogen nevertheless has important roles in male such as regulating male fertility and developing efferent ductile and prostate (Hess & Cooke, 2018). Postmenopausal estrogen deficiency accelerates osteoclastic bone resorption and is most apparent during the first 3 to 5 years (Khosla et al., 2011a). Estrogen deficiency promote resorption of bone due to increased osteoclast lifespan (Cheng et al., 2022; Hughes et al., 1996). Furthermore, deficiency of estrogen promotes elevation of basic multicellular units of the bone cells through increased activation frequency of bone remodeling unit which increases cortical porosity and enlarges the resorption area of trabecular surfaces (Eriksen et al., 1999; Khosla et al., 2011b). In addition, estrogen deficiency can lead to increased

production of inflammatory cytokines such as IL-7 and TNF-α resulting in decreased activity of mature osteoblast cells (Weitzmann et al., 2002). Estrogen also regulates RANK signaling in osteoclastic cells and induces apoptosis of osteoclasts (Cheng et al., 2022; Shevde et al., 2000). Estrogen has protective effect on atherosclerosis progression and are shown to lower plasma lipids and lipoproteins, mediated primarily by changes in hepatic cholesterol metabolism by reducing hepatic acyl-CoA: cholesterol acyltransferase 2 (Kavanagh et al., 2009). Additionally, estrogen acts on endothelium and vascular smooth muscles which leads to decrease in the expression of adhesion molecules involved in monocyte adhesion to endothelial cells and their migration into the subendothelial spaces (Berliner et al., 1990; Xing et al., 2009). Furthermore, estrogen may promote vasodilation by altering vascular reactivity by opening calcium activated potassium channels in smooth muscle cells (White et al., 1995; Xing et al., 2009). Hence, estrogen has protective role on both osteoporosis and atherosclerosis iinflammatory markers.

2.7.7 Inflammatory markers

Cytokines such as interleukin-6 (IL-6), tumor necrosis factor (TNF-α) and C-reactive proteins (CRP) are inflammatory markers that are known to be associated with both atherosclerosis (Russell, 1999; Sulkava et al., 2017) and osteoporosis (Mundy, 2007). Studies indicate that increased level of inflammatory cytokines during chronic inflammation promotes osteoclast differentiation and bone tissue destruction by inhibiting osteoblast maturation (Rao et al., 2018). Higher serum level of IL-6 is responsible for osteoclastogenesis and increased trabecular bone resorption (Lim et al., 2016; M; Papanicolaou et al., 1998). Cytokines may promote proliferation of the endothelial and smooth muscle cell, ease LDL transport through increased permeability of the endothelial cells and differentiation of smooth muscle cells, thus, promoting their growth, proliferation, and migration. CRP contribute to the atherosclerotic process by mediating monocyte recruitment and by stimulating monocytes to release cytokines such as IL-6, and TNF-α (Ilesanmi-Oyelere et al., 2019; Verma et al., 2006). Hence, a systemic inflammatory process could be common mechanism for the development of atherosclerosis and osteoporosis.

2.7.8 Homocysteine

Homocysteine is a variant of cysteine, produced when methionine is broken down in the body (Raisz, 2004). Elevated level of homocysteine promote hardening of arteries and venous thrombosis (Moll & Varga, 2015). Furthermore, homocysteine promote the proliferation of vascular smooth muscle cells, impaired endothelial function, and increase lipid oxidation (Boushey et al., 1995). Increase homocysteine level in blood palsma modulates osteoclast formation via increased reactive oxygen species which cause bone matrix degradation and decrease in blood flow in bone cells (Behera et al., 2017) thereby, inducing osteoblast apoptosis. Osteoporotic bone fracture and decrease in bone mineral density is associated with elevated levels of plasma homocystine level (Raisz, 2004; Vacek et al., 2013).

2.7.9 Hypertension

Hypertension, or high blood pressure, is associated with major cause of morbidity and mortality worldwide (Mills et al., 2020; Zhou et al., 2021). High blood pressure effects the artery walls through direct mechanical pressure, which over time cause endothelial dysfunction (Liu et al., 2022). This leads to deposition of LDL cholesterol in the arterial wall which in turn facilitated formation of atherosclerotic lesion. Furthermore, hypertension also induces oxidative stress in the arterial wall which may induce inflammatory response leading to the formation of atherosclerotic plaque (Cachofeiro et al., 2009). Presence of high blood pressure is linked with increased bone loss and may contribute to the risk of osteoporotic fracture (Cappuccio et al., 1999; J. Zhang et al., 2015). Hypertension alter calcium metabolism leading to increased calcium loss from bone which may accelerate osteoporosis (Ye et al., 2017).

2.7.10 Diabete mellitus

Diabetes mellitus is a metabolic disorder characterized by elevated blood glucose levels. Type 1 and type 2 diabetes are the main subtype of diabetes. In diabetes mellitus 1 the body's immune system attacks and destroys the cells that produce insulin whereas in type 2 diabetes the body does not produce enough insulin. Type 1 diabetic mellitus usually occurs at young age when bone mass has not yet reached its peak bone and hence, could increase the risk of developing osteoporosis (Dunger & Acerini, 1998; N. Napoli & Conte, 2022) and atherosclerosis early in life (Dahl-Jørgensen et al., 2005). In type 1 diabetes mellitus and in the later stages of type 2

diabetes mellitus, there is decrease in osteoblast activity due to inhibitory effect of insulin on osteoblasts, either directly or through alterations in insulin-like growth factor 1 levels (Napoli et al., 2017). Elevated glucose level in blood induces the formation of advanced glycation end products which affect osteoblast proliferation and differentiation via the activation of necrosis factor-kB, resulting in an increased expression of cytokines, growth factors, and adhesion molecules and thus, contributing to the activation of inflammatory processes linked to bone remodeling disorder (Cipriani et al., 2020; Hein, 2006). Advanced glycation end products also stimulate reactive oxygen species production and activate the endothelium and surface expression of adhesion molecules, thereby promoting the adhesion of monocytes/macrophages into the subendothelial space and hence promoting the progression of pathogenic inflammation in atherosclerosis (Poznyak et al., 2020; Yuan et al., 2019).

2.7.11 Bone morphogenic protein

Bone morphogenic protein is a member of transforming growth factor beta superfamily that are known to play important role in the development and maintenance of adipose, neurological, cardiovascular, pulmonary, gastrointestinal, urinary, and musculoskeletal systems Bone morphogenic protein are known to enhance osteoblastic differentiation and proliferation resulting in bone formation (Carreira et al., 2015; Huntley et al., 2019). Furthermore, when stimulated, bone morphogenic protein also express osteoblast genes such as alkaline phosphatase, collagen I, and osteocalcin which are essential for bone formation (Farhat & Cauley, 2008). Expression of bone morphogenic proteins is upregulated in human atherosclerotic plaque (Dhore et al., 2001; Oksala et al., 2010). Bone morphogenic protein stimulates reactive oxygen species in the endothelial cells, which in turn promote the differentiation of smooth muscles and may contribute to vascular calcification (Dhore et al., 2001). Furthermore, inhibition of bone morphogenic protein reduce progression of atherosclerosis lesion by reducing LDL levels, indicating a role of bone morphogenic protein in LDL cholesterol metabolism (Derwall et al., 2012).

2.7.12 Osteoprotegerin

OPG, a member of tumour necrosis factor receptor superfamily, is an important marker of bone remodeling which plays an important role in pathogenesis of both atherosclerosis and osteoporosis. OPG, produced by osteoblast, acts as a decoy receptor for RANKL and regulates osteoclastogenesis by blocking the interactions of RANKL with RANK (Lacey et al., 1998), thereby reducing the osteoclastic bone resorption (Bouchareychas & Raffai, 2018; Boyce & Xing, 2008; Simonet et al., 1997). High levels of OPG were significantly and independently associated with the severity and 10-year development of carotid atherosclerosis, incident cardiovascular disease, and vascular mortality (Kiechl et al., 2004). OPG enhances the expression of endothelial adhesion molecules which result in infiltration of leukocytes and monocytic cells resulting in the progression of carotid atherosclerosis (Dutka et al., 2022; Zauli et al., 2007). However, there are also studies suggesting the protective nature of OPG (Bennett et al., 2006; Bucay et al., 1998; Morony et al., 2008).

2.7.13 Lifestyle factors

Lifestyle factors such as physical activity, smoking and alcohol intake are related to development of both atherosclerosis and osteoporosis. Nicotine and carbon monoxide in cigarette smoke can cause endothelial cell dysfunction, monocyte differentiation into macrophages and formation of foam cell (Sprini et al., 2014). Toxins from smoke alter bone homeostasis by altering osteoclast and osteoblast activity (Al-Bashaireh et al., 2018). Toxins from smoke are also responsible for lower bone mass via dysfunctional gastrointestinal absorption of calcium and vitamin D(Cusano, 2015). Furthermore, studies have indicated that children of parents who smoke have impaired bone health in their adulthood (Juonala et al., 2019) . Physical activity is reported to stimulate bone growth and preserve bone mass (Tolonen et al., 2018) hence, improving balance, and reducing the risk of fall (Alghadir et al., 2015). Furthermore, physical activity increases muscle activity which leads to increase in the blood flow in the bone cells which stimulates the osteoblastic osteogenesis and decreases osteoclast (Benedetti et al., 2018). Physical activity improves lipid metabolism, reduces insulin sensitivity, and have positive effects on

the vascular structure through an increase of nitric oxide bioavailability to improve endothelial function (Chen et al., 2022; Thijssen et al., 2010). Furthermore, excessive alcohol use can increase blood pressure, blood cholesterol levels, reduce estrogen level, and weaken immune response which are major risk factors for both atherosclerosis and osteoporosis (Goncalves et al., 2015; Turner & Sibonga, 2001).

2.7.14 Genetic Factors

Osteoporosis is highly heritable with heritability ranging from 50% to 80% (Zdravkovic et al., 2002; Zhu & Zheng, 2021). However, heritability varies widely among atherosclerosis traits (Zdravkovic et al., 2002) ranging from 58 % for coronary artery calcification score to 78% for coronary calcified plaque volume (Drobni et al., 2022). The *osteoprotegerin*, apolipoprotein *E*(*ApoE*) and low-density lipoprotein receptor related protein (*LRP*) genes have been indicated as possible genetic determinants of atherosclerosis and osteoporosis (Farhat & Cauley, 2008). Mice that are deficient in *osteoprotegerin* gene exhibit decrease in total bone density characterized by severe trabecular and cortical bone porosity and increase in vascular calcification in the aorta and renal arteries suggesting that *osteoprotegerin* may have role in pathogenesis of atherosclerosis and osteoporosis (Bucay et al., 1998; Kostenuik & Shalhoub, 2005). *LRP* gene family encodes cell surface receptors with over 10 members of this gene family. *LRP5* and *LRP6* have been classified as a key mediator of BMD with an important role in bone homeostasis (Lara-Castillo & Johnson, 2015). *LRP5* is needed for later stages of differentiation while *LRP6* is needed for early stages of differentiation of osteoblast (Riddle et al., 2013). *LRP6* play an important role in regulation of vascular smooth muscle cell proliferation (Labbé & Thorin, 2019) while *LRP5* are involved in the differentiation of monocyte to macrophage suggesting its migratory function in atherosclerotic lesions (Borrell-Pagès et al., 2014; Borrell-Pages et al., 2014). *ApoE* genotype has been associated with both atherosclerosis and osteoporosis and helps to transport cholesterol and lipids into lymph system and then into the blood. Specifically, *apoE4*, one of major *apoE* isoforms, is linked with higher LDL cholesterol, (Davignon, 2005; Marais, 2021) which encourage uptake of macrophage and smooth muscle cells, thus accelerating systemic inflammation and promoting atherosclerotic plaque formation (Davignon et al., 1999; Fazio et al., 1997). *ApoE*4 may be associated with decrease in

bone formation leading to increased risk of osteoporosis and bone fractures (Johnston et al., 1999; Souza et al., 2018).

2.8 Omics

Advances in high-throughput molecular profiling technologies have revolutionized the biomedical research, shifting the traditional individual molecule-wise study to system-level studies. These technologies have made it possible to profile a huge number of molecules within a cell or tissue. The scientific fields associated with characterization of a certain type of molecule such as genes, proteins or metabolites from a cell or tissue in its whole is broadly called as "omics". Transcriptomics, genomics, proteomics, metabolomics, lipidomics and methylomics are few examples that are categorized as omics, which correspond to comprehensive analyses of RNA, genes, proteins, metabolites, lipids, and methylated DNA respectively (Micheel et al., 2012). This study is specifically based on transcriptomics and lipidomics.

Transcription, the initial stage of gene expression, is the process by which a gene is converted into protein-coding or non-coding regulatory RNA molecules with the help of an enzyme called RNA polymerase. The complete set of RNA molecules transcribed in a cell or a tissue including ribosomal RNA (rRNA), messenger RNA (mRNA), transfer RNA (tRNA), microRNA (miRNA), and other non-coding RNA (ncRNA), is called the transcriptome. The next step of gene expression involves decoding mRNA to create a specific amino acid chain with the help of ribosomes. The amino acid chain then folds to form an active protein and carries out its functions in the cell. Therefore, transcriptome profile of a cell or a tissue provides information about its biological state. For example, changes in the mRNA level of a transcript identified in the transcriptome of a group of people with certain disease as compared to healthy controls indicates that changes in the mRNA level might have led to altered protein turn out, thus affecting its biological function leading to the

disease. Therefore, transcriptome profiling is crucial for studying the molecular mechanisms of diseases and to identify diagnostic or prognostic markers of diseases. Two major technologies for transcriptome profiling are microarrays and RNA sequencing (RNAseq). Microarrays are microscopic slides containing thousands of tiny spots at specific locations containing DNA molecules serving as probes to measure gene expression (Lockhart et al., 1996). The probes hybridize to specific RNA transcripts and generate fluorescence to determine the relative expression level of the target genes, the intensity of the fluorescence produced by the probe-target RNA transcript hybridization is measured. The basic assumption is that probe-target RNA transcript hybridization levels are proportional to gene expression levels. A major limitation of microarray technology is that only the genes which have probes available in the microarray can be measured, thus restricting the study to only known genes. RNAseq, a more recent technology than microarrays, is a sequencing technique used for transcription profiling that requires no DNA probes and can directly sequence transcripts. As compared to microarrays, RNAseq is more specificity and sensitive in detecting transcripts allowing detection of novel transcripts, allele-specific expression, and splice junctions. Consequently, microarrays are rapidly being replaced by RNAseq for transcriptome profiling. Transcriptomic data used in this study was based on Illumina microarray technology 'Illumina HumanHT-12 version 4 Expression BeadChip'.

The metabolome is the complete set of small-molecule metabolites found within a biological sample such as a cell, tissue or biofluid. Lipidomics, a sub-field of metabolomics, is a complete set of cellular lipid content within a biological sample (J. Wang et al., 2019). As lipids play crucial role in human health and disease, the ability provided by lipidomics to be able to investigate entire spectrum of molecular lipids is of paramount to identify marker lipid species associated with diseases with lipidome-wide association study. The lipidome data used in this study was generated using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

3 AIMS OF THE STUDY

Atherosclerosis related diseases and osteoporosis share several common risk factors and are both associated with significant healthcare costs globally. However, despite the strong evidence for co/multimorbidity hypothesis of these diseases in existing literature, studies investigating joint underlying molecular mechanisms of the diseases using omics data, such as transcriptomics and lipidomic, are lacking. Therefore, the overall aim of this study was to identify molecular and system-level multiomic biomarkers jointly associated with early markers or traits of atherosclerosis and osteoporosis. The specific aims of the study were:

1. To develop weighted co-expression network based co/multimorbidity analysis pipeline and identify plasma lipidomic architecture jointly shared by early markers of atherosclerosis and osteoporosis. (Study I)

2. To identify whole blood transcriptomic architecture jointly shared by early markers of atherosclerosis and osteoporosis. (Study II)

3. To identify plasma molecular lipidomic markers jointly shared by early markers of atherosclerosis and osteoporosis. (Study III)

4. To identify biological processes underlying atherosclerosis-osteoporosis co/multimorbidity. (Study IV)

4 MATERALS AND METHODS

4.1 Material and methods

4.1.1 Study participants

This study was based on the Cardiovascular Risk in Young Finns study (YFS), a national longitudinal multicenter study initiated in 1980 with 3,596 participants aged 3–18 years. The participants were randomly selected among six age cohorts from the areas of five university hospitals in Finland (Turku, Tampere, Helsinki, Kuopio, and Oulu) and have been followed up for eight waves over 40 years after the baseline in 1980 to investigate impact of childhood risk factors for cardiometabolic outcomes in adulthood (Raitakari et al., 2008). The baseline and follow-up studies included comprehensive data collection using questionnaires, physical measurements, dietary interviews, and blood tests from childhood to young adulthood and midlife.

In this thesis, I included the participants who attended the YFS 27-year follow-up in 2007 and had simultaneous measurements of early markers of both osteoporosis and atherosclerosis, covariates as well as lipidomic (I and III) and transcriptomics data (II and IV) as follows:

Study I was based on 1,494 YFS participants aged 30–45 years from the 2007 follow-up with plasma lipidomic data, four early markers for atherosclerosis based on carotid artery ultrasound and six for osteoporosis based on peripheral quantitative computed tomography (pQCT).

Study II was based on 1,032 participants, aged 30–45 years, from the 2007 follow-up, with four atherosclerotic and six osteoporotic early marker data and blood transcriptomic data analysed profiled from the participants during the YFS 2011 follow-up.

Study III was based on 1,545 YFS participants aged 30–45 years from the 2007 follow-up study, with plasma lipidome data, one carotid ultrasound based atherosclerotic (CIMT) and two pQCT based bone density markers.

Study IV included 1,093 YFS participants, aged 31-45 years, from the 2007 follow-up with one atherosclerotic and two osteoporotic early markers. Blood transcriptomic data for this study was profiled from the participants during the 2011 YFS follow-up.

4.1.2 Ethical considerations

The study was approved by the ethical committee of the Hospital District of Southwest Finland on 20 June 2017 (ETMK:68/1801/2017) and Regional Ethics Committee of the Expert Responsibility area of Tampere University Hospital, Helsinki University Hospital Ethical Committee of Medicine, The Research Ethics Committee of the Northern Savo Hospital District and Ethics Committee of the Northern Ostrobothnia Hospital District. The study protocol of each study phase corresponded to the WHO proposal. All the present participants gave written informed consent, and the study was conducted in accordance with the Helsinki Declaration. At prior follow-ups of the Young Finns Study, informed consent of every participant under the age of 18 was obtained from a parent and / or legal guardian.

4.1.3 Measurement of early markers of osteoporosis

Two trained researchers in each study center performed the peripheral quantitative computed tomography (pQCT) bone measurements from both the distal and the diaphysis sites of the radius and tibia [Figure 7]. The same pQCT device was used in all five centers (XCT 2000R, Stratec, Medizintechnik, Pforzheim, Germany). The tomographic slices were taken from the shaft (a cortical-rich bone site) and the distal part (a trabecular-rich bone site) of the weight-bearing tibia (30% and 5% from the distal endplate of the tibia, respectively) and of the nonweight-bearing radius (30% and 4% from the distal endplate of the radius, respectively) according to our standard procedures (Laaksonen et al., 2010).

For the shaft regions, the analyzed bone traits were total area (ToA, mm2), cortical area (CoA, mm2), and cortical density (CoD, mg/cm3). For the distal parts of the radius and tibia, the measured bone traits were ToA (mm2), CoA (mm2) and trabecular density (TrD, mg/cm3). Mineral content was calculated as 0.2 x (area/100) x density. Detailed assessment of these bone density and geometrical parameters from pQCT scans has been described in (Laaksonen et al., 2010).

Precision of the pQCT methods in this multicentre study was evaluated by performing repeated scans of volunteers in each centre before starting and after completing the measurements. Radius and tibia were measured among 39 women and men twice with repositioning. Reproducibility (coefficient of variation, CV%) was 0.5% for distal tibia TrD and 1.6% for distal radius TrD. Early markers of osteoporosis used in the statistical analyses in this study were:

i. Distal radius trabecular bone mineral density (mg/cm3) (DRTrD).
ii. Distal tibia trabecular bone mineral density (mg/cm3) (DTTrD).
iii. Distal radius total bone mineral content (mg) (DRToBMC).
iv. Radial shaft cortical bone mineral content (mg) (RSCoBMC).
v. Distal tibia total bone mineral content (mg) (DTToBMC).
vi. Tibia shaft cortical bone mineral content (mg) (TSCoBMC).

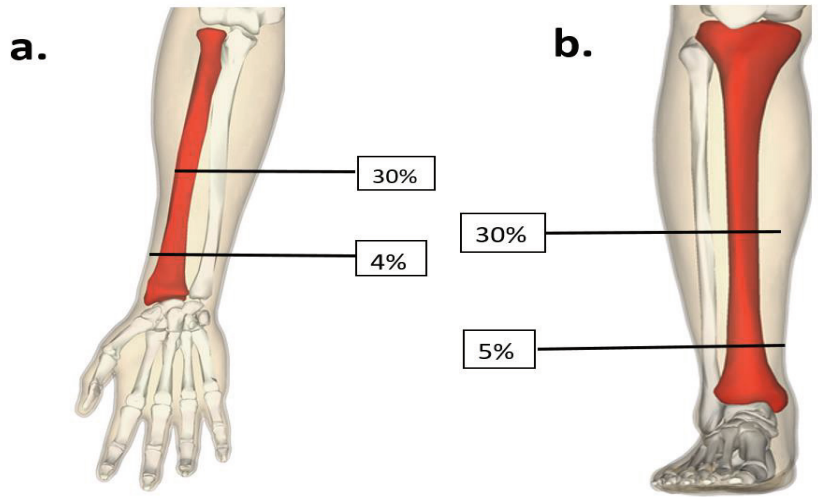

Figure 7. Typical (a) radius and (b) tibia using peripheral quantitative computed tomography with scan location indicated as percent of the length of the respective bones. Picture modified from "BodyParts3D, © The Database Center for Life Science licensed under CC Attribution-Share Alike 2.1 Japan."via Wikimedia Commons

4.1.4 Measurement of early markers of atherosclerosis

Carotid and bulbus intima-media thickness (IMT) were used as surrogate markers of subclinical atherosclerosis [Figure 8]. An ultrasound imaging device with a high-resolution system (Sequoia 512, Acuson) including 13.0 MHz linear array transducers was used for IMT measurement by trained sonographers following a standardized protocol. The image was focused on the posterior (far) wall, and images were recorded from the angle showing the greatest distance between the lumen–intima interface and the media–adventitia interface. A scan including the beginning of the carotid bifurcation and the common carotid artery was recorded and stored in digital format on optical discs for subsequent off-line analysis. All scans were analyzed by one reader blinded to the participants' details. The best- quality end-diastolic frame was selected. Several measurements of the common carotid far wall was taken approximately 10 mm proximally to derive the maximal carotid IMT. To assess the reproducibility of the IMT measurements, we re-examined 60 participants 3 months after the initial visit (2.5% random sample). The between-visit coefficient of variation of IMT measurements was 6.4%. To assess the reproducibility of the IMT image analysis, 113 scans were re-analyzed by a second observer, and the coefficient of variation was 5.2%. The mean and maximum carotid and bulbus IMT was used in the study. Early markers of atherosclerosis used in the statistical analyses in this study were:

i. Carotid intima-media thickness (average, mm) (CIMTavg).
ii. Carotid intima-media thickness (maximum, mm) (CIMTmax).
iii. Bulbus intima-media thickness (average, mm) (BIMTavg).
iv. Bulbus intima-media thickness (maximum, mm) (BIMTmax).

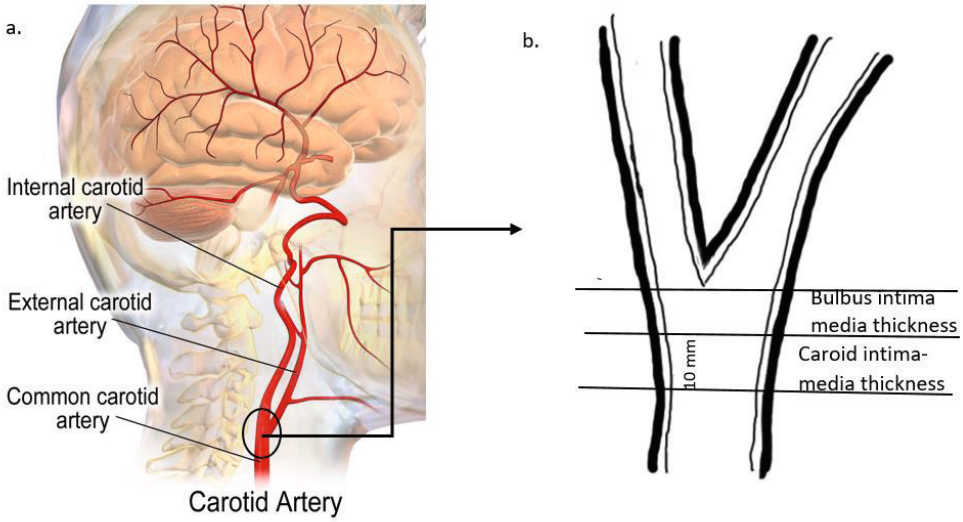

Figure 8. a. Illustration of location of measurement of early markers of atherosclerosis. Figure b shows schematic diagram of the longitudinal view of carotid artery. The measurements are performed in beginning of the carotid bifurcation (BIMTavg and max) and approximately 10 mm proximally of the common carotid artery (CIMTavg and max). Modified from: Blausen.com staff (2014). "Medical gallery of Blausen Medical 2014". *WikiJournal of Medicine* 1 (2). DOI:10.15347/wjm/2014.010. ISSN 2002-4436

4.1.5 Clinical characteristics and laboratory measurements

Weight of the YFS participants was measured in light clothes without shoes using a digital scale with 0.1 kg accuracy. Height of the participants was measured by a wall-mounted stadiometer (Karhu, Finland) with the accuracy of 0,5 cm. BMI was calculated as weight in kilograms (kg) divided by the square of height in meters (m^2). Blood pressure was measured from the right-side brachial artery with a random-zero sphygmomanometer (Hawksley & Sons Ltd.; Lancing, UK) in 2007, and in 2011. Blood pressure was measured in the sitting position after a five minute's rest. Korotkoff's fifth phase was used as the sign of diastolic blood pressure and the first phase as the sign of systolic blood pressure. Readings to the nearest even number of millimeters of mercury were performed at least thrice on each subject. The statistical analyses were based on the average of these three measurements.

Venous samples were drawn from the right antecubital vein after a 12-hours overnight fast. In the adulthood follow-ups conducted in 2007, and 2011, serum or plasma was separated and stored at -70 °C until the time of analysis. In 2007 and 2011, all the analyses were performed in the laboratory for the Population Research of the National Institute for Health and Welfare, Turku. The serum total cholesterol and triglyceride concentrations were determined enzymatically (Olympus System Reagent; Olympus Diagnostica GmbH, Hamburg, Germany) in a clinical chemistry analyzer (AU400; Olympus Optical Ltd, Mishima, Japan). HDL cholesterol was analyzed after the precipitation of very low-density lipoprotein cholesterol and LDL cholesterol with dextran sulphate-$MgCl_2$. The concentration of LDL cholesterol was calculated using the Friedewald formula for participants with triglycerides <4,0 mmol/l. In 2007, and 2011, serum insulin was measured using the microparticle enzyme immunoassay kit (Abbott Laboratories, Diagnostic Division, Dainabot, Japan). The serum glucose concentrations were determined by the enzymatic hexokinase method (Glucose System Reagent, Beckman Coulter Biomedical O'Callaghan's Mills, Ireland) on an automatic analyzer (AU400, Olympus, Tokyo, Japan).

4.1.6 Lifestyle data

Physical activity index, based on weekly metabolic equivalent hours (MET-h/wk.), was calculated from information on the frequency, intensity and duration of physical activity including leisure-time physical activity and commuting to the workplace. One MET corresponds to the energy consumption of one kilocalorie per kilogram of weight per hour at rest (Pälve et al., 2014).

Cigarette smoking was ascertained as a part of standardized self-administered questionnaire. Data on smoking status was dichotomized into smokers and non-smokers. Individuals who reported daily smoking were defined as smokers. Individuals who reported smoking less often than daily or never were defined as non-smokers.

Participants' alcohol consumption information was also based on self-administered questionnaire on their alcohol consumption during the previous week where one unit is equivalent to 14 g of alcohol (Juonala et al., 2009). Skewness in the values for body mass index (BMI), physical activity and alcohol consumption was corrected with log2 transformation.

4.1.7 Assessment of dietary calcium and vitamin D

In 2007, information on food consumption and nutrient intake was collected with a modified 131- item FFQ developed by the Finnish National Institute for Health and Welfare (Paalanen et al., 2006). The nutrient contents of reported foods were calculated using the Finnish Food Composition database, Fineli® (National Institute for Health and Welfare). Calcium (mg/day) and vitamin D (µg/day) were presented as a mean intake/day.

4.1.8 Plasma lipidomic profiling

Lipidome quantification for the stored plasma samples was performed at Zora Biosciences Oy (Espoo, Finland). In brief, 10 µl of 10 mM 2,6-di-tert-butyl-4-methylphenol in methanol was added to 10 µl of sample, followed by 20 µl of internal standards (Avanti Polar Lipids Inc., Alabaster, AL) and 300 µl of chloroform:methanol (2:1, v:v) (Sigma-Aldrich GmbH, Steinheim, Germany). Samples were mixed and sonicated in a water bath for 10 min, followed by a 40-min incubation and centrifugation (15 min at 5700 ×*g*). The upper phase was transferred and evaporated under nitrogen. Extracted lipids were resuspended in 100 µl of water saturated butanol and sonicated in a water bath for 5 min. 100 µl of methanol was added to the samples before the extracts were centrifuged for 5 min at 3500 ×*g*, and finally the supernatants were transferred to the analysis plate for mass spectrometric (MS) analysis. The analyses were performed on a hybrid triple quadrupole/linear ion trap mass spectrometer (QTRAP 5500, AB Sciex, Concords, Canada) equipped with ultra-high-performance liquid chromatography (Nexera-X2, Shimadzu, Kyoto, Japan). Chromatographic separation of the lipidomic screening platform was performed on Acquity BEH C18, 2.1 × 50 mm id. 1.7 µm column (Waters Corporation, Milford, MA, USA). The data were collected using a scheduled multiple reaction monitoring algorithm and the data were processed using Analyst and MultiQuant 3.0 software (AB Sciex). The heights of the peaks obtained from the MS

analysis were normalized with the internal standard of the lipid classes. Skewness in the lipid profiles were corrected with log transformation of the data.

4.1.9 Blood transcriptomics profiling

Whole-genome transcriptome was profiled from whole-blood samples collected from the YFS participants during the 2011 follow-up. Expression levels were analysed with Illumina HumanHT-12 version 4 Expression BeadChip (Illumina Inc.), containing 47,231 expression and 770 control probes. Samples with fewer than 6,000 significantly detected expression probes (detection $p<0.01$) were discarded. Raw Illumina summary probe-level data was exported from Beadstudio and processed in R (http://www.r-project.org/) using a nonparametric background correction, followed by quantile normalization with control and expression probes, with the neqc function in the limma package (Ritchie et al., 2015) and a log2 transformation. Nine samples were excluded due to sex mismatch between the recorded sex and predicted sex based on RPS4Y1-2 and XIST mRNA levels on the Y and X chromosomes, respectively. After that, expression data were available for 1,654 samples, including 4 technical replicates, which were used to examine batch effects and subsequently excluded before further analysis.

4.2 Biostatistical analysis

For this study, I combined existing biostatistical methods for weighted gene co-expression network analysis, MANOVA and biological pathway enrichment analysis as a new approach for investigation of shared molecular basis for different co/multimorbidities using omic data. All the statistical analyses in this study were performed using the R environment for statistical computing, versions 3.4.3, 3.6.0 and 3.6.1 (R Development Core Team, 2019).

4.2.1 Omic network module identification with weighted co-expression network analysis

In studies I and II, we applied co-expression network analysis method implemented in R statistical software package WGCNA (Langfelder & Horvath, 2008) to identify groups of densely interconnected modules from the YFS lipidomic and transcriptomic data, called as lipid and gene modules respectively [Figure 9]. Molecular lipids in case of lipidomic data or genes in case of transcriptomic were referred as nodes and correlation between the nodes were referred as connection or edges of a co-expression network. The process of module identification involved calculation of Pearson's correlation (r) for all pairwise comparisons of lipids or genes across all participants, resulting in a correlation matrix. Pairs of nodes with positive correlations were considered connected, leading to a signed network. The correlation matrix was transformed to an adjacency matrix by raising it to the power of 5 in case of lipidomic and 10 in case of transcriptomic data. The goal of the power transformation was to minimize noise and emphasize stronger correlations. The powers were chosen using the power function implemented in the WGCNA package such that it transforms the correlation matrix to an approximately scale-free topology based on the assumption that most of the real-world biological networks are scale-free. The resulting adjacency matrix was used to generate Topological Overlap Matrix (TOM), a pairwise similarity matrix that incorporates topological similarity of molecular lipids or genes in the definition of the co-expression of molecular lipids or genes. For example, a high topological overlap implies that a pair of lipid species or genes shares several neighbour lipid species or genes with similar levels. The TOM was transformed into a dissimilarity matrix by subtracting the values in the matrix from 1 (1-TOM). Average linkage hierarchical clustering of the dissimilarity matrix was performed to generate a hierarchical clustering tree of lipid species or genes. Next, lipid species or gene modules were identified with a dynamic tree-cutting algorithm. The quality of the identified modules was further assessed by analysing the correlation between gene significance (GS) or lipid significance (LS) and module membership (MM). GS or LS is defined as the correlation between the module's member genes or lipid species and the study traits. MM is defined as the correlation between the summary expression profile of a module and its member genes or lipid species. An ideal module is one where GS or LS and MM are highly correlated, which suggests that the genes/lipid species that are highly correlated with the biological marker of interest are also the important member of the analysed module.

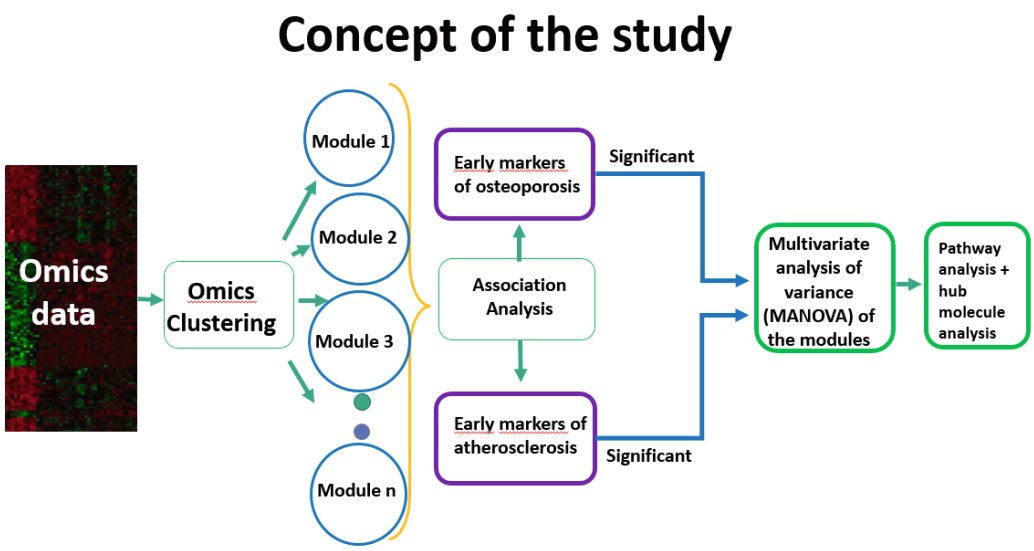

Figure 9. Atherosclerosis-osteoporosis comorbidity biostatistical analysis pipeline.

4.2.2 Association analysis of the identified omics modules and the studied early markers

The first principal component of the expression profiles of the member genes or the lipid species in a module, referred as module eigengenes or eigenlipids (ME) was used as a summary expression profile of the modules. Associations between the lipid or gene modules and the studied traits were assessed by calculating Pearson's correlation coefficients (r) between the modules and the studied markers [Figure 6]. Modules that were significantly correlated (Bonferroni-adjusted p-value) (P_{adj}) < 0.05) with early markers of both atherosclerosis and osteoporosis were considered as candidate modules for testing joint statistical association with the studied early markers of both diseases in multivariate statistical analysis. Multivariate analyses of variance (MANOVA) test implemented in R statistical package car [Fox et al., 2019] was conducted for the significant modules to test for joint statistical association between the candidate modules and the early markers of both atherosclerosis and osteoporosis. In the lipidomic data analysis in study I, the

multivariate analysis was adjusted with age and sex variables. In the transcriptomic data analysis in study II, the multivariate analysis was adjusted with age, sex, BMI, smoking habit, alcohol consumption and physical activity. The Pillai's Trace statistic from MANOVA results represents the magnitude of the effect of the module's summary expression profile on the early traits of atherosclerosis and osteoporosis.The value of the statistic ranges from 0 to 1, with higher values meaning higher effect. The F-value from the MANOVA results represents the predictive ability of a model: a higher F-value suggests higher significance of a model.

4.2.3 Biological pathway enrichment analysis of network modules

In study II, gene modules identified from the transcriptomic data that were jointly associated with early markers of atherosclerosis and osteoporosis were tested for biological pathway (group of biologically related genes) enrichment for their biological interpretation. The enrichment analysis was based on biological pathways derived from Gene Ontology (GO) (Ashburner et al., 2000) , the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Ogata et al., 1999) and the Disease Ontology (DO) (Schriml et al., 2012) using clusterProfiler (Yu et al., 2012) and DOSE (Yu et al., 2015) R/Bioconductor packages.

4.2.4 Multivariate analysis of variance of human plasma lipidome

In study III, we performed MANOVA to investigate lipidome markers jointly associated with early markers of atherosclerosis (CIMTavg) and osteoporosis (DTTrD, DRTrD) in the YFS participants. The analysis was performed using the MANOVA functions implemented in R package car, with early markers of atherosclerosis and osteoporosis as outcomes and lipid species as predictors. Three different models were studied: MANOVA model 1 without any covariates, MANOVA model 2 adjusted for age, sex, and BMI, and MANOVA model 3 adjusted for smoking habit, physical activity, and alcohol consumption in addition to age, sex, and BMI [Table 2]. Under each of the MANOVA models, different outcome combinations, for one atherosclerotic (CIMTavg) and two osteoporosis (DTTrD, DRTrD) early markers were analyzed. The analysis was repeated also for males and females separately. Several lipid species were highly correlated with each other. Therefore, for multiple testing correction, appropriate number of independent

tests was estimated using the eigenvalues of the correlation matrix using the Matrix Spectral Decomposition (matSpDlite) software (Li & Ji, 2005). Multiple testing correction was done by adjusting the p-values with Bonferroni method and lipid species with adjusted p-value (P_{adj}) < 0.05 were considered statistically significant.

Table 2. Multivariate analysis of variance models (MANOVA 1-3) models analyzed in the study.

Model 1	**(***CIMTavg + DTTrD) ~ lipid species*
	(*CIMTavg + DRTrD) ~ lipid species*
Model 2	*(CIMTavg + DTTrD) ~ lipid species + age + sex + BMI*
	(CIMTavg + DRTrD) ~ lipid species + age + sex + BMI
Model 3	*(CIMTavg + DTTrD) ~ lipid species + age + sex + BMI + smoking + alcohol consumption + physical activity*
	(CIMTavg + DRTrD) ~ lipid species + age + sex + BMI + smoking + alcohol consumption + physical activity

Abbreviations: BMI, body mass index; CIMTavg, average carotid intima media thickness; DTTrD, total mineral density of the distal tibia's trabecular bone; DRTrD, distal radius trabecular bone mineral density.

4.2.5 Differential gene expression and gene set analysis of transcriptomic data

In study IV, we performed both differential gene expression (DGE) and gene set analyses of the transcriptomic data to identify genes and biological processes associated with early markers of atherosclerosis and osteoporosis in the YFS

participants. The early markers of the diseases were transformed into binary categorical variable. For the early marker of atherosclerosis, CIMTavg value higher than or equal to 90th percentile was used for the definition of high CIMT. For the early marker of osteoporosis, we calculated the YFS population-based T-scores for trabecular BMD at distal radius (DRTrD) and tibia (DTTrD). The T-score represents the magnitude of deviation of a participant's BMD from BMD of an average healthy 31-45 years old people of the same sex. Participants with T-score ≤ -1 were considered as cases for low BMD as an early indicator of osteoporosis [Laaksonen et al., 2010]. The definition of cases for low BMD was based on both distal tibia and distal radius using the corresponding reference values (Laaksonen et al., 2010) and analysis was repeated for both distal tibia and distal radius-based case-control setting. DGE and gene set analyses were performed on the residuals after performing a regression analysis of the transcriptomic data against age, sex, BMI, physical activity index (MET), smoking habit, alcohol consumption and blood cell counts of erythrocytes, leukocytes, and thrombocytes. DGE analysis concerning early markers of both atherosclerosis and osteoporosis separately was performed using moderated t-test implemented in linear models for microarray data (limma) R/Bioconductor package (Ritchie et al., 2015). Gene set analysis was performed using rotation gene set test (ROAST) (D. Wu et al., 2010) implemented in limma R/Bioconductor package against the latest version of curated gene sets (c2.all.v7.4) downloaded from molecular signatures database (MSigDB) (Liberzon et al., 2015). ROAST is a self-contained gene set test that tests whether any of the genes in the set are differentially expressed (Goeman & Bühlmann, 2007). ROAST is different from other self-contained gene set analysis methods such as SAM-GS (Dinu et al., 2007) in that, instead of permutation of sample labels, ROAST uses rotation, a Monte Carlo technique for multivariate regression, for p-value estimation (Langsrud, 2005). In addition to the analysis of the whole population with mixed sex, we also performed sex-stratified gene set analysis to identify sex-specific associations between the studied gene sets and the studied early markers. We tested potential modification of effect of the identified biological processes on the early markers of atherosclerosis and osteoporosis by sex by analyzing regression models of the studied early markers (in both categorized and continuous forms) against eigengene of the analyzed gene set (summary expression level of a gene set calculated as the first principal component of the member genes), eigengene and sex interaction, sex, age, BMI, smoking and alcohol consumption habit.

5 RESULTS

5.1 Lipidomic results

5.1.1 Identification of lipidomic modules jointly associated with early markers of atherosclerosis and osteoporosis (study I)

5.1.1.1 Study population characteristics

The characteristics of the study participants in study I are shown in Table 3. Disease incidence data was based on self-reports. The early markers of atherosclerosis and osteoporosis used in the statistical analyses of the study are shown in Table 3.

Table 3. Population characteristics of the Young Finns Study (YFS) participants. Data are expressed as mean (± SD) or percentages. Source: study I.

Variable (unit)	**Men**	**Women**
Number of subjects, N (%)	646 (43%)	848 (57%)
Age, years	38 (5)	38 (5)
Body mass index, kg/m^2	26.5 (3.9)	25.1 (4.7)
Total cholesterol (mmol/l)	5.2 (0.9)	4.9 (0.8)
LDL cholesterol (mmol/l)	3.3 (0.8)	3.0 (0.7)
HDL cholesterol (mmol/l)	1.2 (0.3)	1.5 (0.3)
Triglycerides (mmol/l)	1.6 (0.9)	1.2 (0.6)
Serum glucose (mmol/l)	5.5 (0.6)	5.2 (0.7)
Insulin (IU/l)	9.9 (26.3)	8.3 (8.6)
C-reactive protein (mg/l)	1.6 (4.7)	2.0 (3.5)
Systolic blood pressure (mmHg)	125.2 (13.1)	116 (13.4)
Diastolic blood pressure (mmHg)	78.3 (10.9)	72.8 (10.7)
Alcohol consumption, units/day	1.4 (1.9)	0.6 (0.7)
Physical activity index (MET h/wk)	20.4 (22.2)	19.4 (20.1)
Daily smoking, N (%)	129/641 (20%)	121/843 (14%)
Daily calcium intake (mg/day)	1371 (602)	1190 (483)
Daily vitamin D intake (μg/day)	8.4 (4.5)	7.3 (3.5)
Family risk factor for coronary heart disease, N (%)	107/646 (16.6%)	140/847 (16.5%)
Participants with osteoporosis, N (%)	3/641 (0.5%)	8/845 (1%)
Participants with epilepsy, N (%)	5/624 (0.8%)	7/835 (0.8%)
Participants with Crohn's disease, N (%)	5/625 (0.8%)	9/836 (1.1%)
Participants with Anorexia, N (%)	0	8/836 (1%)
Usage of corticosteroids at least once a month, N (%)	13/624 (2.1%)	54/837 (6.5%)

Table 4. Early markers of atherosclerosis and osteoporosis in the Young Finns Study cohort used in study I with their descriptive statistics among the study participants expressed as mean (±SD). Source: modified from study I.

Variable description (unit)	Abbreviations	Mean (±SD)
Early markers of atherosclerosis		
Carotid intima-media thickness (average, mm)	*CIMTavg*	0.6 (0.1)
Carotid intima-media thickness (maximum, mm)	*CIMTmax*	0.7 (0.2)
Bulbus intima-media thickness (average, mm)	*BIMTavg*	0.8 (0.1)
Bulbus intima-media thickness (maximum, mm)	*BIMTmax*	0.8 (0.1)
Early markers of osteoporosis		
Total mineral density of the distal radius's trabecular bone (mg/cm^3)	*DRTrD*	224.4 (36.1)
Total mineral density of the distal tibia's trabecular bone (mg/cm^3)	*DTTrD*	240.3 (34.1)
Total mineral content of the distal radius (mg)	*DRToBMC*	243.6 (64.2)
Total mineral content in the radial shaft's cortical bone (mg)	*RSCoBMC*	214.2 (44.9)
Total mineral content in the distal tibia (mg)	*DTToBMC*	602.1 (126.9)
Total mineral content in the tibia shaft's cortical bone (mg)	*TSCoBMC*	646.4 (110.5)

5.1.1.2 Correlation among the early markers of atherosclerosis and osteoporosis

The early markers of osteoporosis had a weak but significant (p-value < 0.01) positive correlation with those of atherosclerosis [Figure 10].

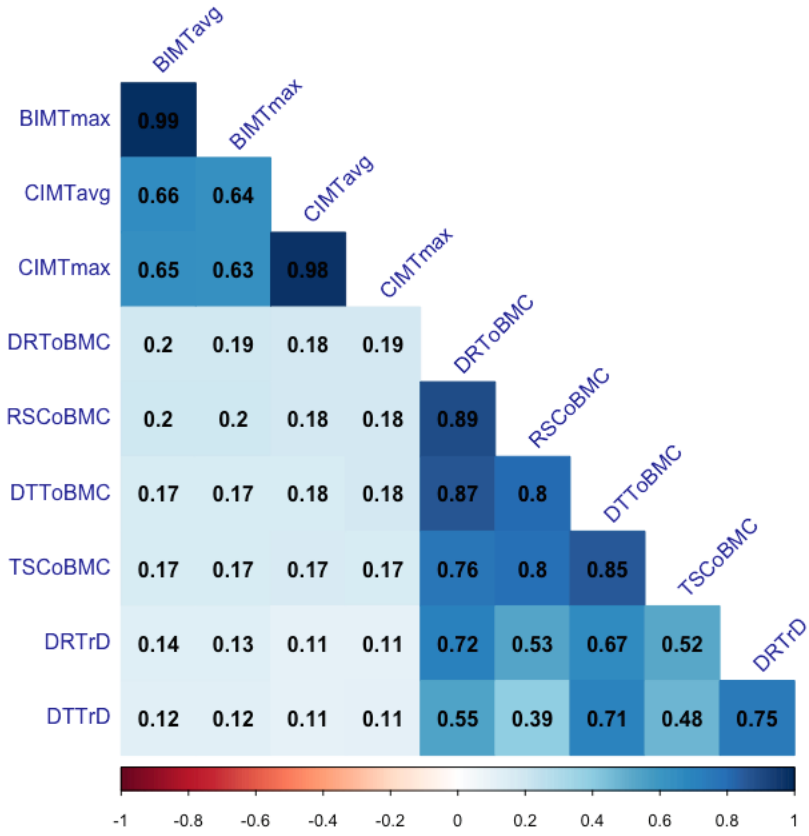

Figure 10. Pearson's correlation coefficients (r) between the early markers of atherosclerosis and osteoporosis. All correlations are statistically significant (p-value < 0.01). The abbreviations in this figure are explained in Table 4. Source: modified from study I.

5.1.1.3 Lipidomic modules jointly associated with the early markers of atherosclerosis and osteoporosis.

Weighted co-expression network analysis [Section 4.2.1] of the lipidome data identified three modules (named turquoise, pink and yellow) significantly associated early markers of both the diseases [Figure 11].

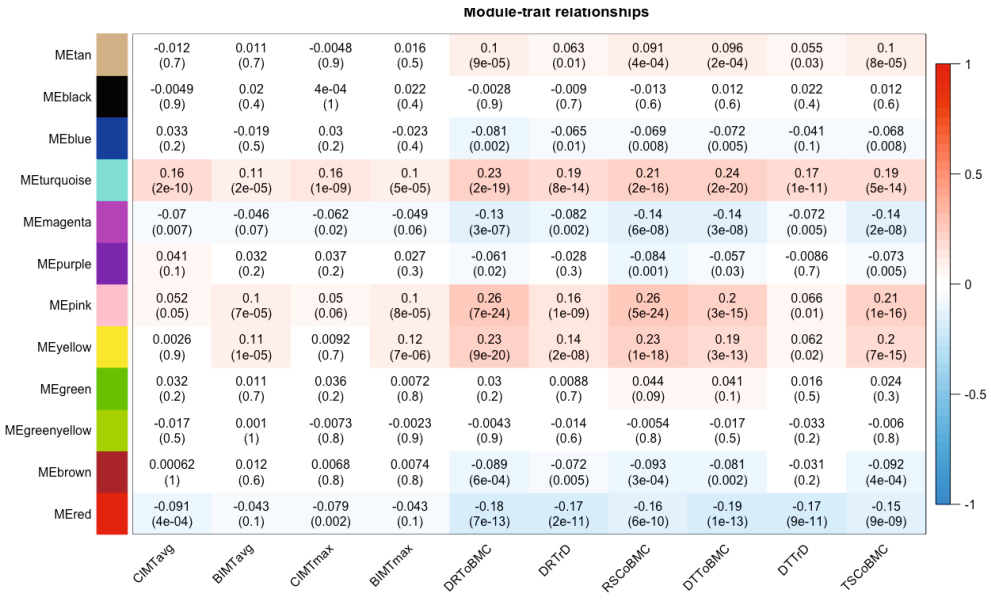

Figure 11. Module–early marker relationships. The rows correspond to the different modules and their eigenlipids (ME). The columns correspond to the early markers of atherosclerosis and osteoporosis used in the study. The values in the cells represent Pearson's correlation coefficients (r), with the associated p-values in parentheses. The modules are named according to colour and the correlation coefficients have a colour-coding shown in the colour legend (between -1 and +1) on the right side of the figure. The abbreviations for the early markers in the column names in the bottom of the figure are explained in Table 4. Source: modified from study I.

The turquoise module was significantly associated with atherosclerosis related variables CIMTavg (r=0.16, p-value=2 x 10-10) and CIMTmax (r=0.16, p-value=1 x 10-9) as well as with osteoporosis related variables DRToBMC (r=0.23, p-value=2 x 10-19), DRTrD (r=0.19, p-value=8 x 10-14), RSCoBMC (r=0.21, p-value=2 x 10-16), DTTrD (r=0.17, p-value=1 x 10-11) and DTToBMC (r=0.24, p-value=2 x 10-20). Similarly, the pink and yellow modules were significantly associated with both bulbus IMT variables BIMTavg (pink: r=0.10, p-value=7 x 10-5, yellow: r=0.11, p-value=1 x 10-5) and BIMTmax (pink: r=0.10, p-value=8 x 10-5, yellow: r=0.12, p-value=7 x 10-6). The same modules were also significantly associated with five of the six early markers of osteoporosis. We further tested the quality of the three modules by assessing the correlation between lipid significance (LS) and module membership (MM) of each of the modules with respect to each of the early markers [Section 4.2.1]. Only the turquoise module had significant correlation between LS

and MM with respect to the early markers of both atherosclerosis (CIMTavg; r=0.66, p-value=1.9 x 10-14) and osteoporosis (DTToBMC; r=0.64, p-value=2 x 10-13) markers [Figure 12]. Therefore, further analysis was based on the turquoise module only.

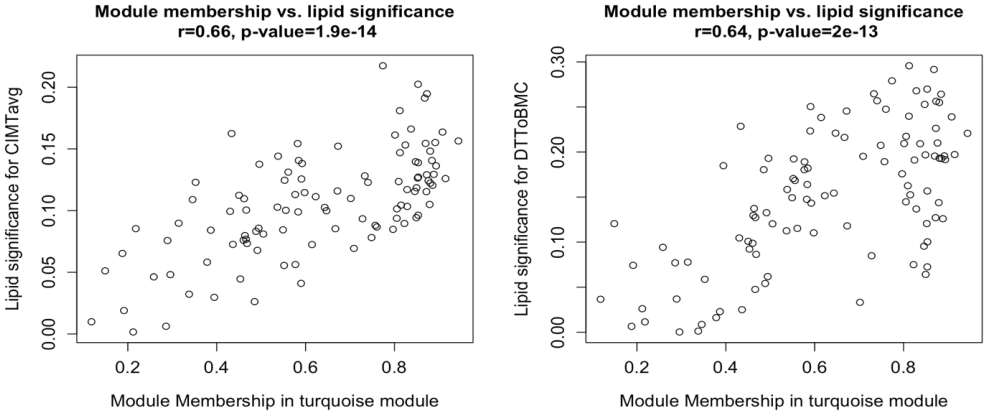

Figure 12. Scatter plots of lipid significance (LS) vs module membership (MM) in the turquoise module. The left panel corresponds to early marker of atherosclerosis and the right panel to osteoporosis. Abbreviations: CIMTavg, carotid intima media thickness (average); bbmax, bulbus intima media thickness (maximum); DTToBMC, total mineral content in distal tibia. Source: modified from study I.

Multivariate analysis of variance (MANOVA) of the turquoise module was done with CIMTavg and DTToBMC as outcomes and eigenlipid of the module as predictor adjusted with age and sex. CIMTavg and DTToBMC were chosen as outcomes in the MANOVA model because those variables had the maximum and the most significance correlation with eigenlipid of the turquoise model [Figure 8]. We found a statistically significant joint association between the turquoise module and the early markers of the diseases, $F(2, 1489)=12.50$, p-value=4.1 x 10-6, Pillais' Trace=0.01.

The turquoise model contained 105 lipid species, majority of which belonged to the classes of glycerolipid, glycerophospholipid and sphingolipid [Figure 13 A].

There were 19 diacylglycerol and 41 triacylglycerol lipid species in the glycerolipid class (Figure 13 B). There were seven phosphatidylcholine lipid species in the glycerophospholipid class and 20 ceramide species in the sphingolipid class.

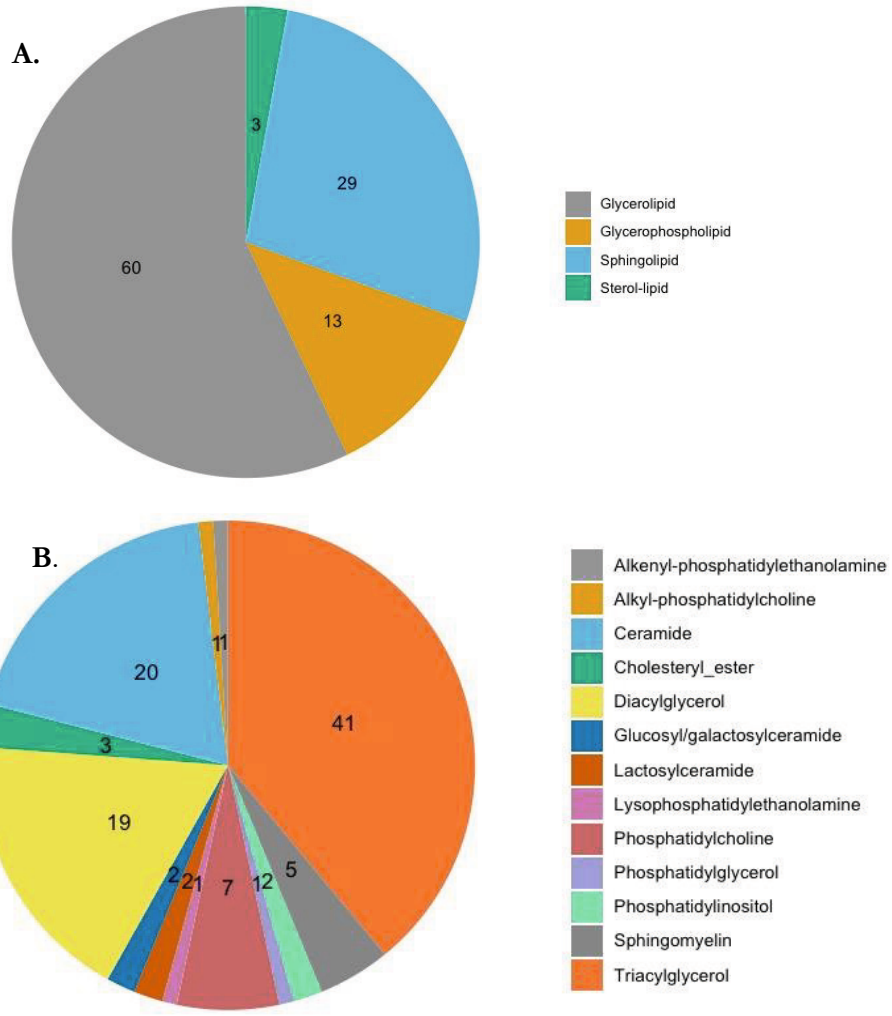

Figure 13. Distribution of lipids classes (A) and constituent lipids (B) in the joint turquoise module for early markers of atherosclerosis and osteoporosis. Source: study I.

5.1.2 Identification of lipidomic markers jointly associated with early markers of atherosclerosis and osteoporosis (study III)

5.1.2.1 Study population characteristics

The characteristics of the study participants and measured early markers of atherosclerosis and osteoporosis used in study III are shown in Table 5. Disease incidence data was based on self-reports.

Table 5. Population characteristics and measured early markers of atherosclerosis and osteoporosis of the Young Finns Study cohort. Data are mean (± standard deviation) or proportions (%). Source: modified from study III.

Variables (unit)	**Men**	**Women**
Number of subjects, N (%)	669 (43 %)	876 (57 %)
Age (years)	38 (5)	38 (5)
Body mass index, kg/m^2	27 (4)	25 (5)
Total cholesterol (mmol/l)	5.2 (0.9)	4.9 (0.8)
LDL cholesterol (mmol/l)	3.3 (0.8)	3.0 (0.7)
HDL cholesterol (mmol/l)	1.2 (0.3)	1.4 (0.3)
Triglycerides (mmol/l)	1.5 (0.7)	1.2 (0.6)
Serum glucose (mmol/l)	5.5 (0.7)	5.2 (0.7)
Insulin (IU/l)	10 (26)	8.5 (8.5)
C-reactive protein (mg/l)	1.7 (4.7)	2 (3.4)
Systolic blood pressure (mmHg)	125 (13)	116 (14)
Diastolic blood pressure (mmHg)	78 (11)	73 (11)
Participants with hypertension, N (%)	40/662 (6 %)	45/876 (5 %)
Alcohol consumption, units/day	1.4 (2)	0.6 (0.7)
Physical activity index (*MET*-h/wk)	20 (20)	20 (22)
Daily smoking, N (%)	129/669 (19%)	119/876 (14%)
Daily calcium intake (mg/day)	1364 (606)	1188 (504)
Daily vitamin D intake (μg/day)	8.4 (4.6)	7.4 (3.6)
Family risk factor for coronary heart disease, N (%)	109/669 (16%)	156/876 (18%)
Participants with osteoporosis, N (%)	3/664 (0.5%)	7/874 (0.8%)
Participants with bone fractures, N (%)	297/647 (46%)	280/866 (32%)
Participants with family history for osteoporosis, N (%)	25/642 (4%)	61/858 (7%)
Usage of corticosteroids at least once a month, N (%)	16/647 (3%)	61/865 (7%)
Carotid intima-media thickness (*CIMTavg*) (average, mm)	0.65 (0.11)	0.61 (0.09)
Participants with *CIMTavg* > 1mm	5/669 (0.7%)	0/876
Distal radius trabecular bone mineral density (DRTrD) (mg/cm^3)	247 (31)	207 (28)
Distal tibia trabecular bone mineral density (DTTrD) (mg/cm^3)	255 (33)	229 (30)

5.1.2.2 Lipidomic markers jointly associated to early markers of atherosclerosis and osteoporosis

We identified four lipid species jointly associated with early markers of atherosclerosis and osteoporosis with statistical significance of $P_{adj} < 0.05$ from MANOVA model 3 [Figure 14]. The four lipid species were TAG(18:0/18:0/18:1) from class of glycerolipids, PC (40:3) from class of glycerophospolipids and Gb3(d18:1/22:0) and Gb3(d18:1/24:0) both from class of sphingolipids. We identified eight lipid species with model 2 adjusted only for age, sex, and BMI. The four lipid species identified from model 3 included four of the eight lipid species identified from model 2. For reference, over 200 lipid species were identified from model 1 without any covariates. We also investigated sex-specific associations between lipid species and early markers of the diseases with MANOVA model adjusted with age, BMI, smoking habit, physical activity, and alcohol consumption. However, no lipid species were identified with statistical significance of $P_{adj} < 0.05$.

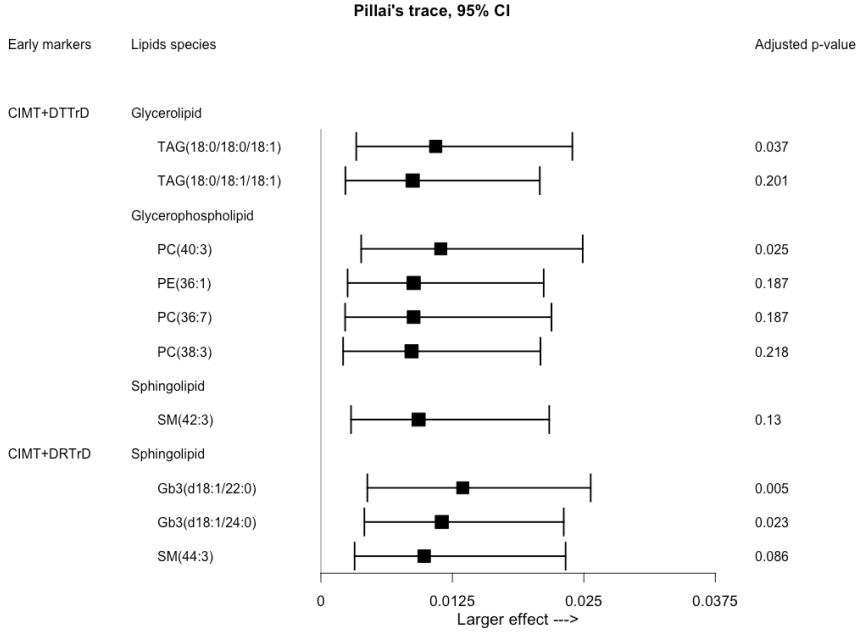

Figure 14. Forest plot of the multivariate lipidome-wide association study of early markers of atherosclerosis and osteoporosis adjusted for age, sex, body mass index, smoking habit, physical activity, and alcohol consumption (MANOVA model 3). Analysis was performed for two different outcomes based on combination of early markers of atherosclerosis,

carotid intima media thickness (CIMTavg); and osteoporosis, total mineral density of the distal tibia's trabecular bone (DTTrD, upper results) and distal radius's trabecular bone (DRTrD, bottom results). The second column represents lipid species and the classes they belong to. Pillai's trace is the test statistic in multivariate analysis of variance (MANOVA) ranging from 0 to 1. The 95% confidence interval (95% CI) of the test statistic was calculated from 100 bootstraps of the original data. P-values were adjusted using Bonferroni's method. Source: study III.

5.2 Transcriptomic results

5.2.1 Identification of transcriptomic modules jointly associated with early markers of atherosclerosis and osteoporosis (study II)

5.2.1.1 Study population characteristics

The characteristics of the study participants and measured early markers of atherosclerosis and osteoporosis used in study II are shown in Table 6. The measured early markers of atherosclerosis and osteoporosis are shown in Table 7. The gene expression levels of the study participants were profiled in 2011 follow-up. Disease incidence data was based on self-reports.

Table 6. Population characteristics of the participants in study II. Data are expressed as means (± SD) or proportions (%). Source: study II.

Variables (unit)	**Men**	**Women**
Number of subjects, N (%)	454 (44%)	578 (56%)
Age (years)	38 (5)	38 (5)
Body mass index (kg/m$^{2)}$	26.5 (4)	25 (4.7)
Total cholesterol (mmol/l)	5.2 (0.9)	4.9 (0.8)
LDL cholesterol (mmol/l)	3.3 (0.8)	3.0 (0.7)
HDL cholesterol (mmol/l)	1.2 (0.3)	1.5 (0.3)
Triglycerides (mmol/l)	1.5 (0.7)	1.1 (0.5)
Serum glucose (mmol/l)	5.4 (0.5)	5.2 (0.7)
Insulin (IU/l)	10.4 (31.2)	8.0 (7.6)
C-reactive protein (mg/l)	1.7 (5.5)	1.9 (3.3)
Systolic blood pressure (mmHg)	124.8 (13.3)	116 (13.5)
Diastolic blood pressure (mmHg)	78.1 (11.1)	72.6 (10.9)
Alcohol consumption (units/day)	1.4 (2.1)	0.6 (0.7)
Physical activity index (MET-h/wk.)	20.5 (22.70)	19.1 (20)
Daily smoking, N (%)	77/453 (17%)	70/576 (12%)
Daily calcium intake (mg/day)	1393 (613)	1174 (453)
Daily vitamin D intake (μg/day)	8.6 (4.6)	7.4 (3.4)
Family risk factor for coronary heart disease, N (%)	72/454 (15.9%)	97/578 (16.8%)
Participants with osteoporosis, N (%)	3/451 (0.7%)	6/577 (1%)
Participants with epilepsy, N (%)	3/441 (0.7%)	4/573 (0.7%)
Participants with Crohn's disease, N (%)	3/442 (0.7%)	5/573 (0.9%)
Participants with anorexia, N (%)	0	5/573 (0.9%)
Usage of corticosteroids at least once a month, N (%)	7/442 (2%)	37/573 (6%)
Participants with type 1 diabetes, N (%)	1/450 (0.2%)	4/578 (0.7%)
Participants with type 2 diabetes, N (%)	3/449 (0.7%)	3/578 (0.5%)
Participants with menopause, N (%)	-	0/578 (0%)

Table 7. Early markers of atherosclerosis and osteoporosis with their descriptive statistics among the study participants in study II expressed as mean (± SD). Source: study II.

Variable description (unit)	Acronym	Mean± SD
Early traits of atherosclerosis		
Carotid intima-media thickness (average, mm)	*CIMTavg*	0.63 (0.10)
Carotid intima-media thickness (maximum, mm)	*CIMTmax*	0.66 (0.11)
Bulbus intima-media thickness (average, mm)	*BIMTavg*	0.80 (0.14)
Bulbus intima-media thickness (maximum, mm)	*BIMTmax*	0.83 (0.14)
Early traits of osteoporosis		
Distal radius trabecular bone mineral density (mg/cm^3)	*DRTrD*	225 (36)
Distal tibia trabecular bone mineral density (mg/cm^3)	*DTTrD*	241 (34)
Distal radius total bone mineral content (mg)	*DRToBMC*	245 (65)
Radial shaft cortical bone mineral content (mg)	*RSCoBMC*	215 (45)
Distal tibia total bone mineral content (mg)	*DTToBMC*	605 (127)
Tibia shaft cortical bone mineral content (mg)	*TSCoBMC*	651 (110)

5.2.1.2 Transcriptomic modules jointly associated with the early markers of atherosclerosis and osteoporosis

Weighted co-expression network analysis (Section 4.2.1) of the transcriptomic data identified six gene modules named by colours (brown, honeydew, darkseagreen, lightcoral, green and tan) significantly associated early markers of both the diseases [Figure 15].

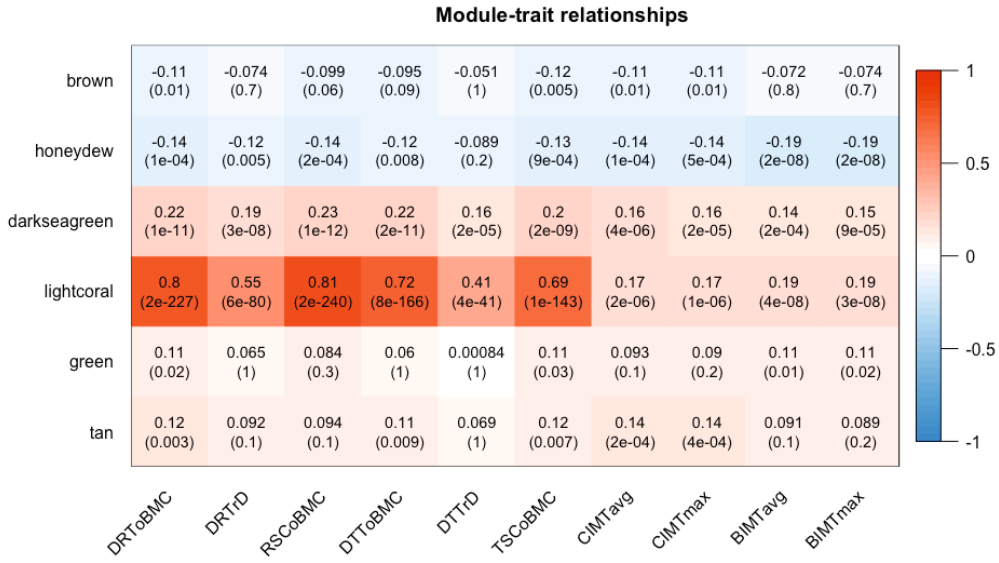

Figure 15. Module–early marker relationships.The row names correspond to the different modules and their eigengenes (ME). The column names correspond to the early markers of atherosclerosis and osteoporosis used in the study. The values in the cells represent Pearson's correlation coefficients (r), with the associated p-values in parentheses. The modules are named according to color and the correlation coefficients have a color-coding shown in the color legend (between -1 and +1) on the right side of the Figure. The abbreviations for the early markers in the column names in the bottom of the figure are explained in Table 4. Source: Study II.

MANOVA test was conducted for the six gene modules to test their joint association with early markers of both the diseases. The test was adjusted for age, sex, BMI, smoking, alcohol consumption and physical activity. The results are summarized in Table 8. Two of the six significant modules, honeydew and green, were jointly associated with early traits of both atherosclerosis and osteoporosis with $P_{adj} \leq 0.03$.

Table 8. Multivariate analysis of variance (MANOVA) test results from association analyses between the summary expression profiles of the six significant gene modules and early traits of both diseases. atherosclerosis and osteoporosis adjusting for age, sex, body mass index, smoking, alcohol consumption and physical activity. Source: Study II.

Gene module names (number of genes)	**Pillai's Trace**	**F-value**	**P_{adj*}**
brown (1,205)	0.002	1.09	0.34
***honeydew* (33)**	0.007	4.0	**0.03**
darkseagreen (28)	0.003	2.0	0.19
lightcoral (13)	0.0004	0.2	0.84
***green* (3,885)**	0.008	4.0	**0.02**
tan (504)	0.003	2.0	0.18

Abbreviations: P_{adj}, Bonferroni-adjusted p-value.

5.2.1.3 Multivariate analysis of the member genes of the gene modules jointly associated with the early markers of atherosclerosis and osteoporosis

We performed MANOVA for the member genes of the two gene modules (honeydew and green) that were jointly associated with the early markers of the diseases. We identified Nitric Oxide Synthase Interacting Protein (*NOSIP*) as the most significant gene with P_{adj} of 0.09 in the honeydew module. Similarly, we identified tripartite motif containing 63 (*TRIM63*) as the most significant gene in the green module with P_{adj} of 0.07.

5.2.1.4 Pathway analysis of the gene modules jointly associated with the early markers of atherosclerosis and osteoporosis

Genes in the honeydew module were significantly enriched with two GO based biological processes [Table 9], both of which were related to the immune response. The green module was enriched with six GO pathways (4 biological processes and 2 molecular functions) and one KEGG pathway [Table 9]. The pathways are related to olfactory receptors and signal transduction.

Table 9. Pathways enriched in honeydew and green gene co-expression modules.

Gene modules	**ID**	**Description**	p_{adj}*	**Categories**
honeydew	GO:0042110	T cell activation	0.005	Biological process
	GO:0051251	positive regulation of lymphocyte activation	0.05	Biological process
green	GO:0050911	detection of chemical stimulus involved in sensory perception of smell	0.0009	Biological process
	GO:0007608	sensory perception of smell	0.001	Biological process
	GO:0009593	detection of chemical stimulus	0.002	Biological process
	GO:0007156	homophilic cell adhesion via plasma membrane adhesion molecules	0.007	Biological process
	GO:0004984	olfactory receptor activity	7.6×10^{-5}	Molecular function
	GO:0048018	receptor ligand activity	0.04	Molecular function
	hsa04740	Olfactory transduction	8.9×10^{-5}	KEGG

Abbreviations: P_{adj}*: Bonferroni adjusted p-value.

5.2.2 Identification of genes and biological processes jointly associated with early markers of atherosclerosis and osteoporosis (study IV)

5.2.2.1 Study population characteristics

Population characteristics and summary statistics of the early markers of atherosclerosis and osteoporosis of the studied population in study IV are shown in Tables 10, 11 and 12. Number of diseases are based on self-reports. Both high CIMT and low BMD were significantly associated with age, BMI, HDL-cholesterol, triglycerides, and insulin.

Table 10. Population characteristics of the Young Finns study participants in study IV concerning subclinical atherosclerosis (high CIMT). Participants with high CIMT (> 90th percentile) were defined as cases and the rest as controls. Data are expressed as mean (± SD) or proportions (%) and statistical significance (p-value) of the difference between the cases and controls.

Variable (unit)	Cases	Controls	P-value
Number of subjects (%)	107 (10 %)	986 (90 %)	-
Sex (female %)	34 %	58 %	-
Age (years)	41 (4)	38 (5)	< 0.0001
Body mass index (kg/m^2)	28 (5)	26 (5)	< 0.0001
Total cholesterol (mmol/l)	5.2 (1)	5.0 (0.9)	0.15
LDL cholesterol (mmol/l)	3.2 (0.9)	3.1 (0.8)	0.11
HDL cholesterol (mmol/l)	1.2 (0.3)	1.4 (0.3)	< 0.0001
Triglycerides (mmol/l)	1.7 (1)	1.3 (0.9)	0.001
Serum glucose (mmol/l)	5.6 (0.9)	5.3 (0.9)	0.003
Insulin (IU/l)	12.9 (14.9)	8.5 (7.4)	0.003
C-reactive protein (mg/l)	2.5 (9.6)	1.8 (3.3)	0.43
Systolic blood pressure (mmHg)	129 (15)	119 (14)	< 0.0001
Diastolic blood pressure (mmHg)	81 (12)	75 (11)	< 0.0001
Participants with hypertension (%)	17/106 (16%)	46/984 (5 %)	< 0.0001
Alcohol consumption (units/day)	1.1 (1.5)	0.9 (1.5)	0.25

Physical activity index (MET-h/wk)	18 (19)	20 (22)	0.38
Daily smoking (%)	15/107 (14 %)	136/986 (14 %)	1
Daily calcium intake (mg)	1314 (606)	1256 (526)	0.34
Daily vitamin D intake (μg)	8.5 (4.7)	7.8 (4)	0.18
Family risk factor for coronary heart disease (%)	26/107 (24 %)	154/986 (16 %)	0.03
Participants with osteoporosis (%)	2/106 (2 %)	7/983 (1 %)	0.49
Participants with bone fractures (%)	43/107 (40 %)	368/986 (37 %)	0.63
Participants with family history for osteoporosis (%)	6/107 (6 %)	51/977 (5 %)	1
Usage of corticosteroids at least once a month (%)	7/107 (7 %)	49/986 (5 %)	0.64
Carotid intima-media thickness (CIMT) (average, mm)	0.84(0.08)	0.61(0.07)	-
Participants with CIMT > 1 mm	5/107 (5 %)	0/986	-
Distal radius trabecular bone mineral density (DRTrD) (mg/cm^3)	234 (36)	225 (36)	0.009
Distal tibia trabecular bone mineral density (DTTrD) (mg/cm^3)	251 (34)	240 (34)	0.003

Table 11. Population characteristics of the Young Finns study participants concerning low bone mineral density (low BMD) of distal tibia as marker of subclinical osteoporosis. The Young Finns study population-based T-scores for trabecular BMD from distal tibia were calculated and T-score ≤ -1 was used to define cases (low BMD). Data are expressed as mean (± SD) or proportions (%) and statistical significance (p-value) of the difference between the cases and controls.

Variable (units)	Cases	Controls	P-value
Number of subjects (%)	176 (16 %)	917 (84 %)	-
Sex (female %)	60 %	45 %	-
Age (years)	39 (5)	38 (5)	0.01
Body mass index (kg/m^2)	24(4)	26 (5)	< 0.0001
Total cholesterol (mmol/l)	5.1 (0.9)	5.0 (0.9)	0.12
LDL cholesterol (mmol/l)	3.1 (0.8)	3.1 (0.8)	0.7
HDL cholesterol (mmol/l)	1.4 (0.3)	1.3 (0.3)	< 0.0001
Triglycerides (mmol/l)	1.3 (0.7)	1.4 (0.9)	0.05
Serum glucose (mmol/l)	5.3 (1)	5.3 (0.9)	0.69
Insulin (IU/l)	7.1 (6.2)	9.3 (8.9)	0.0001
C-reactive protein (mg/l)	1.4 (2.4)	1.9 (4.6)	0.04

Systolic blood pressure (mmHg)	119 (15)	120 (14)	0.32
Diastolic blood pressure (mmHg)	74 (12)	76 (11)	0.09
Participants with hypertension (%)	7/175 (4 %)	56/915 (6 %)	0.35
Alcohol consumption (units/day)	1.0 (1.4)	0.9 (1.5)	0.3
Physical activity index (MET-h/wk.)	16 (18)	20 (22)	0.002
Daily smoking (%)	35/176 (20 %)	116/917 (13 %)	0.02
Daily calcium intake (mg)	1181 (489)	1277 (542)	0.02
Daily vitamin D intake (μg)	7.8 (4.2)	7.9 (4)	0.75
Family risk factor for coronary heart disease (%)	35/176 (20 %)	145/917 (16 %)	0.22
Participants with osteoporosis (%)	3/175 (2 %)	6/914 (1 %)	0.34
Participants with bone fractures (%)	70/176 (40 %)	341/917 (37 %)	0.25
Participants with family history for osteoporosis (%)	10/174 (6 %)	47/910 (5 %)	0.91
Usage of corticosteroids at least once a month (%)	11/176 (6 %)	45/917 (5 %)	0.58
Carotid intima-media thickness (CIMT) (average, mm)	0.62(0.1)	0.63 (0.1)	0.46
Participants with CIMT > 1 mm	2/176 (1 %)	3/917 (0.5 %)	0.4
Distal radius trabecular bone mineral density (DRTrD) (mg/cm^3)	195 (27)	232 (35)	< 0.0001
Distal tibia trabecular bone mineral density (DTTrD) (mg/cm^3)	195 (15)	250 (30)	< 0.0001

Table 12. Population characteristics of the Young Finns study participants concerning low bone mineral density (low BMD) of distal radius as marker of subclinical osteoporosis. The Young Finns study population-based T-scores for trabecular BMD from distal radius were calculated and T-score ≤ -1 was used to define cases (low BMD). Data are expressed as mean (± SD) or proportions (%) and statistical significance (p-value) of the difference between the cases and controls.

Variable (unit)	Cases	Controls	P-value
Number of subjects (%)	161 (15 %)	932 (85 %)	-
Sex (female %)	55 %	56 %	-
Age (years)	38 (5)	38 (5)	0.68

Body mass index (kg/m2)	25 (4)	26 (5)	0.0002
Total cholesterol (mmol/l)	5 (0.9)	5 (0.9)	0.34
LDL cholesterol (mmol/l)	3 (0.8)	3.1 (0.8)	0.37
HDL cholesterol (mmol/l)	1.4 (0.3)	1.3 (0.3)	0.11
Triglycerides (mmol/l)	1.2 (0.7)	1.4 (0.9)	0.02
Serum glucose (mmol/l)	5.3 (1)	5.3 (0.9)	0.41
Insulin (IU/l)	7.4 (6.9)	9.1 (8.8)	0.03
C-reactive protein (mg/l)	1.4 (2.1)	1.9 (4.6)	0.02
Systolic blood pressure (mmHg)	118 (14)	121 (14)	0.04
Diastolic blood pressure (mmHg)	74 (11)	76 (11)	0.02
Participants with hypertension (%)	7/161 (4 %)	56/929 (6 %)	0.51
Alcohol consumption (units/day)	1 (1.7)	0.9 (1.5)	0.81
Physical activity index (MET-h/wk)	21 (20)	19 (22)	0.37
Daily smoking (%)	26/161 (16 %)	125/932 (13 %)	0.42
Daily calcium intake (mg)	1212 (470)	1270 (545)	0.16
Daily vitamin D intake (mg)	7.9 (4.2)	7.9 (4)	0.95
Family risk factor for coronary heart disease (%)	23/161 (14 %)	157/932 (17 %)	0.49
Participants with osteoporosis (%)	4/161 (2 %)	5/928 (0.5 %)	0.04
Participants with bone fractures (%)	74/161 (46 %)	337/932 (36 %)	0.02
Participants with family history for osteoporosis (%)	11/159 (7 %)	46/925 (5 %)	0.42
Usage of corticosteroids at least once a month (%)	11/161 (7 %)	45/932 (5 %)	0.38
Carotid intima-media thickness (CIMT) (average, mm)	0.62(0.1)	0.63(0.1)	0.3
Participants with CIMT > 1 mm	0/161	5/932 (0.5 %)	0.76
Distal radius trabecular bone mineral density (DRTrD) (mg/cm3)	182 (21)	233 (33)	<2.2 x 10-16
Distal tibia trabecular bone mineral density (DTTrD) (mg/cm3)	211 (27)	247 (33)	<2.2 x 10–16

5.2.2.2 Identification of genes jointly associated with early markers of atherosclerosis and osteoporosis

In study IV, the traditional univariate DGE analysis of the transcriptomics data identified two genes, *RDH8* (retinol dehydrogenase 8) and *CFAP74* (cilia and flagella associated protein 74) to be associated with high CIMT both with FDR of 0.04. However, no genes were identified to be associated with low BMD with FDR < 0.05 with the same approach. Multivariate analysis of the transcriptomics data with early markers of both the diseases as outcome did not yield any genes jointly associated with the analysed early markers.

5.2.2.3 Identification of biological processes jointly associated with early markers of atherosclerosis and osteoporosis

Gene set analysis of the transcriptomic data with 6290 curated gene sets (c2.all.v7.4) downloaded from MSigDB identified three novel gene sets [Figure 16] and replicated 234 gene sets significantly associated with high CIMT with FDR ≤ 0.01. Genes in the three novel gene sets had decreased average expression level among participants with high CIMT as compared to controls [Figure 16].

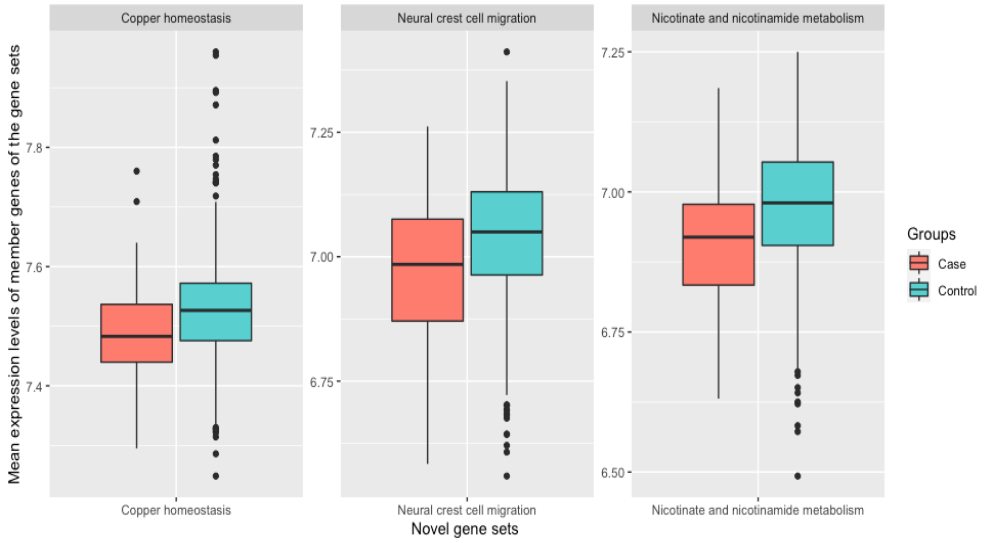

Figure 16. Boxplots illustrating difference in mean expression levels of member genes of the three novel gene sets associated with high carotid intima media thickness (cases, CIMT > 90th percentile) as compared with controls. The middle "box" represents the middle 50% of mean expression levels of the cases and controls. The upper and lower whiskers represent expression levels outside the middle 50%. The dots represent outliers. Source: Study IV.

The replicated 234 gene sets represented different biological processes such as immune system, apoptosis, hypoxia, lipid metabolism, signal transduction and cancer [Figure 17]. However, we did not identify any gene sets associated with reduced trabecular BMD with FDR < 0.05 with either of the distal tibia and distal radius-based case-control analyses.

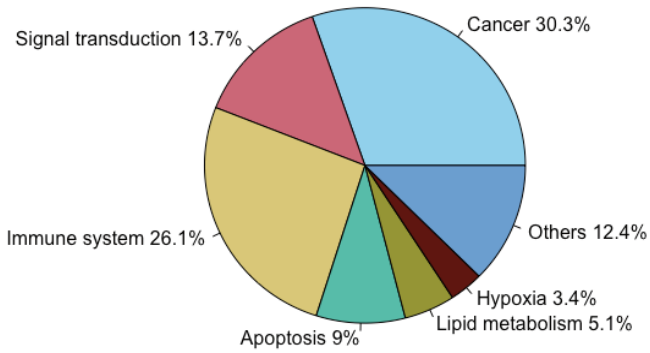

Figure 17. Pie chart summarizing biological processes represented by the 234 gene sets replicated in this study. These gene sets are significantly associated with high CIMT with FDR ≤ 0.01. The category 'Others' includes gene sets involved in pentose phosphate pathway, proteoglycan biosynthesis, beta alanine metabolism, copy number variation, galactose catabolism, vitamin C ascorbate metabolism, pantothenate and CoA biosynthesis, necrosis, and cellular processes. Source: Study IV.

6 DISCUSSION

6.1 Lipidomics (studies I and III)

In the lipidomic based studies, we identified molecular lipids and their modules jointly associated with the early markers of atherosclerosis and osteoporosis. Majority of the lipid species from the significant module identified in study I belonged to glycerolipids, glycerophospholipids and sphingolipids classes. Lysophosphatidylcholine (LPC), a glycerophospholipid, is a pro-inflammatory lipid generated by various pathological activities and a major component of oxidized LDL (Schmitz & Ruebsaamen, 2010).

Oxidized LDL is known to be a potential risk factor for vascular calcification with loss of bone mass (Parhami et al., 1997). Oxidized LDL exhibits many atherogenic activities including endothelial dysfunction, monocyte differentiation, macrophage activation, foam cell formation, smooth muscle cell migration and proliferation which also stimulate the formation of atherosclerotic plaque (Khatana et al., 2020; Stiko-Rahm et al., 1992). Furthermore, studies have also suggested that oxidized LDL inhibit osteoblastic differentiation by inhibition of RANKL signaling pathway (Mazière et al., 2009) and alter osteoblastic cell proliferation, migration, and apoptosis through oxidative stress, and thus, affecting bone metabolism equilibrium (Hamel et al., 2008).

In study I, the three most significant lipid species (TAG(18:0/18:0/18:1), TAG(18:0/18:1/18:1) and TAG(16:0/18:0/18:1)) based on MANOVA of individual lipid species belonging to the turquoise module were triglycerides from glycerolipids. Elevated levels of triglycerides are known to be important predictors in the development of cardiovascular disease (Farnier et al., 2021). Lipoproteins that carry triglycerides in the blood stream induce endothelial dysfunction and adhesion molecule expression macrophages to form foam cells that contribute to the buildup of atherosclerotic plaque along the walls of artery (Bleda et al., 2016; L. Wang et al., 2009). Several studies have also shown that higher triglyceride levels are associated with greater risk of osteoporosis (Bijelic et al., 2016;

Kan et al., 2021; Orozco, 2004). Higher concentration of triglyceride in serum is positively associated with bone marrow fat (Bredella et al., 2013), which leads to lower trabecular bone mineral density in older adults (Schwartz et al., 2013). For example, TAG (54:2), triacylglyceride with fatty acyls of 54 carbons and 2 double bonds has been shown to be a strong predictor for cardiovascular disease (Stegemann et al., 2014). Similarly, TAG (56:6), triacylglyceride with fatty acyls of 56 carbons and 6 double bonds, has been shown to be associated with development of cardiovascular disease in type 2 diabetes mellitus (Alshehry et al., 2016). Osteoporotic tissue cells exhibit higher osteoclastogenesis and lower TAG metabolism suggesting protective role of TAG in osteoporosis (Dragojevič et al., 2013).

It is important to recognize that while a system-level approach provides more statistical power for identifying novel lipidomic modules and hub lipid markers within the molecular modules, it can potentially miss to identify individual lipid species that do not cluster well with other lipid species, perhaps due to their independent biological role in atherosclerosis-osteoporosis comorbidity. Therefore, in this complementary study (study III), we aimed to investigate atherosclerosis-osteoporosis comorbidity hypothesis by performing lipidome-wide multivariate association analysis of early markers of both the diseases to identify molecular lipid species with potentially distinct biological role in the comorbidity. We identified four lipid species, TAG(18:0/18:0/18:1) from class glycerolipid, PC(40:3) from class glycerophospolipid and Gb3(d18:1/22:0) and Gb3(d18:1/24:0) from class sphingolipid, jointly associated with early markers of both diseases.

Sphingolipids are involved in important biological and cellular processes, such as tissue development, cell recognition, proliferation, migration, and apoptosis (Hannun & Obeid, 2018). Sphingomyelins, the most common sphingolipids in mammalian cells and tissues are independently associated with atherosclerosis burden (Jiang et al., 2000; Schlitt et al., 2006). Sphingomyelin is catalyzed by sphingomyelinases to produce ceramide (Hornemann & Worgall, 2013). Ceramide accumulates in atherosclerotic plaques and stimulates the atherosclerosis process (Chatterjee et al., 1997) by promoting LDL infiltration and aggregation due to endothelial dysfunction within the intima of artery walls. Furthermore, ceramides are responsible for activation of NF-ϰB (nuclear factor kappa-light-chain-enhancer of activated B cells) that causes apoptosis of bone cell (Kitajima et al., 1996). Fabry disease, an X-linked disorder of glycosphingolipid metabolism which leads to

accumulation of globotriaosylceramide in body cell. Build-up of globotriaosylceramide triggers immune response, oxidative stress, inflammation (Biancini et al., 2012) and endothelial dysfunction (Satoh, 2014) in Fabry patients. People with Fabry disease have higher rates of osteoporosis and osteopenia and accelerated bone resorption of the lumbar spine and femoral neck (Mersebach et al., 2007; Sacre et al., 2010). Phosphatidylcholine (PC) comprises 40–50% of total cellular phospholipids and play role in cell signaling, food and energy storage and glycerophospholipid metabolism (van der Veen et al., 2017). Phosphatidylcholine and lysophosphatidylcholines, a metabolite of PCs, are likely to increase reactive oxygen species which leads to oxidative stress damage (Zhao et al., 2018). This increase in oxidative stress induces the apoptosis of osteoblasts and osteocytes and thereby exacerbating the process of osteoporosis (Huh et al., 2006; Sánchez-Rodríguez et al., 2007). Studies have shown that phophatidylcholine with lower number of carbon atoms might be associated with cardiovascular disease (Djekic et al., 2019). For example, PC(34:1), a phosphatidylcholine with 34 carbons and 1 double bonds is related to ischemia (Sousa et al., 2016).

6.2 Transcriptomics (studies II and IV)

In study II, we identified two transcriptomic modules jointly associated with the studied early markers of the two diseases. The three most significant genes from the two modules, *NOSIP*, *GXYLT2* and *TRIM63* were jointly associated with the early markers of the studied diseases also individually. NOSIP is related to the metabolism of nitric oxide, which plays a crucial role in the pathogenesis of both osteoporosis (Wimalawansa, 2010) and atherosclerosis (C. Napoli et al., 2006). Nitric oxide regulates blood pressure and vascular tone, inhibits platelet aggregation and leukocyte adhesion, and prevents vascular smooth muscle cells proliferation (C. Napoli et al., 2006). A moderate concentration of nitric oxide can act as a stimulator of osteoblast growth and differentiation, while high concentration of nitric oxide inhibits bone resorption and formation (Wimalawansa, 2010). Effects of nitric oxide on bone tissue are dependent on its concentration. Nitric oxide has dichotomous biological effects, at low concentrations (pico-nano molar range), nitric oxide may promote proliferation, differentiation, and survival of osteoblasts, whereas at high concentrations (micromolar range) nitric oxide may inhibit bone resorption and formation.

GXYLT2 encodes for xylosyltransferase which plays role in biosynthesis of glycosaminoglycan chains, an important constituent of proteoglycans. Proteoglycans are among several extracellular matrix molecules that congregate in atherosclerosis lesions (Wight & Merrilees, 2004) and regulates osteolytic process (Lamoureux et al., 2007). The third significant gene, *TRIM63* (tripartite motif containing 63) also known as *MURF1* (muscle-specific ring finger protein 1), has been shown to promote osteoblastic cell differentiation and suppress proliferation of osteoblastic cells (Azuma et al., 2010). However, the role of *TRIM63* in atherosclerosis is unclear.

In study II, green and honeydew module were jointly and significantly associated with the studied traits of both diseases after adjustment with age, sex, body mass index, smoking habit, alcohol consumption and physical exercise. The genes in honeydew module modules were enriched with biological process such as T-cell activation and positive regulation of lymphocyte activation to be enriched in honeydew module. Activated T-cell contributes to the initiation and progression of atherosclerosis (Bäck et al., 2019) and osteoclastogenesis (Pietschmann et al., 2016). B-lymphocytes are known to regulate the RANK-RANKL-OPG pathway that plays a role in basal bone homeostasis, osteoclast formation and the regulation of bone resorption (Walsh & Choi, 2014). B cells produces cytokines and immunoglobulins that are important regulators of inflammation in atherosclerosis plaque formation (Kyaw et al., 2011).

Genes in green module were enriched with biological processes related to sensory perception of smell and chemical stimulus mostly contained olfactory receptor genes that belong to the G-protein-coupled receptor gene family. Although olfactory receptor genes are traditionally known for odor recognition in olfactory sensory neurons (Feldmesser et al., 2006), various studies have shown diverse physiological functions of olfactory receptors in other tissues, such as the testes (Spehr et al., 2003), lungs (Gu et al., 2014), the brain (Otaki et al., 2004 and the heart (S. H. Kim et al., 2015). Studies have indicated that olfactory receptors may be involved as a metabolic regulator of heart function (Jovancevic et al., 2017) and lipid metabolism (C. Wu et al., 2017). Additionally, bone morphogenetic protein, regulator of bone formation and repair (Carreira et al., 2015) are suggested to promote the survival of olfactory receptors' neurons (Shou et al., 2000).

In study IV, gene set analysis of whole-blood transcriptomic data from the YFS participants identified three novel gene sets representing three biological processes, copper homeostasis, neural crest cell migration and nicotinate and nicotinamide metabolism, associated with early markers of atherosclerosis but not with that of osteoporosis. Copper is an essential trace metal micronutrient that stimulates oxidative modifications of LDL-cholesterol and participate in the oxidation of LDL within the arterial walls which is then transformed into foam cell in the artery wall which is the hallmark of atherosclerosis. Alterations in copper homeostasis can lead to dyslipidemia (L. & Lutsenko, 2013) which plays role in both osteoporosis (Poiana et al., 2013) and atherosclerosis (Linton et al., 2019). Furthermore, elevated serum copper has been shown to be associated with increased serum concentrations of total cholesterol and HDL cholesterol (Song et al., 2018).

Nicotinamide is a potent antioxidant involved in the production of nicotinamide adenine dinucleotide which is responsible for maintaining redox homeostasis and modulating the immune response (X. Zhang et al., 2021). Antioxidants are responsible for the maintenance of the redox hemostasis by elimination of reactive oxygen species (He et al., 2017). Reactive oxygen species molecules trigger oxidative stress that promote endothelial dysfunction by promoting a vascular inflammatory response which leads to the progression of atherosclerosis (Kondo et al., 2009). Oxidative stress may also facilitate extensive bone loss and bone fragility and thereby exacerbating the process of osteoporosis (Domazetovic, 2017).

Neural crest cell is a multipotent cell population that migrates to generate diverse differentiated cell types such as coronary artery smooth muscles cells, skeletal and connective tissue components depending on their axial level of origin and migratory pathways (Vickaryous & Hall, 2006). For example, neural crest cell from the postotic hindbrain region migrate into the heart and differentiate into coronary artery smooth muscle cells (Miyagawa-Tomita et al., 2016). Preotic neural crest cells are capable of osteogenic and chondrogenic differentiation and hence, might be related to the pathogenesis and progression of coronary artery diseases (Miyagawa-Tomita et al., 2016) and osteoporosis (Dash & Trainor, 2020). Alterations in activities of genes involved in neural crest cells migration among participants with high CIMT, as identified in this study, support the hypothesis that neural crest cells may play role in atherosclerosis.

6.3 Limitations of the study

There were certain limitations to this study. The study was based on a relatively young population cohort and therefore limited to the early markers of the diseases with only few clinically diagnosed cases of cardiovascular disease and osteoporosis. Therefore, it is not possible to draw direct conclusions on the associations of these shared early biomarkers of osteoporosis and atherosclerosis in their clinical manifestations. The carotid artery and bone measurements were taken from the 2007 follow-up; however, the transcriptomic data was profiled from samples collected during the 2011 follow-up. Therefore, the study was based on assumptions that there is no substantial change (in the order of magnitude between the participants in 2007 and 2011) in bone and carotid artery traits (Russo et al., 2003; Stein et al., 2004; Uusi-Rasi et al., 2007) over a four-year period among a healthy adult population. Furthermore, all study participants are of European origin and further research in a case–control setting with population groups of different ethnical background is needed.

6.4 Future perspectives

Co/multimorbidities are associated with increased mortality, disability, lower quality of life, extended hospital stays and consequently higher healthcare costs worldwide (Barnett et al., 2012; Whitty et al., 2020). It is crucial and therefore a priority to take co/multimorbidities into account during treatment of an index disease for optimal care and consequently improved clinical outcomes (MacMahon et al. 2018). Still, most health systems around the world are designed to focus on single health conditions and therefore there is serious coordination problem in treating patients with multiple conditions in more holistic way. This study shifts the focus of current biomedical research from single disease framework to spectrum of multiple disease that may co-exist and share common molecular background.

Development of holistic approach to treat patients with multimorbidity requires in-depth knowledge of shared risk factors, clinical parameters and molecular networks underlying the multiple conditions. While there has been several co/multimorbidity studies concerning shared lifestyle risk and clinical factors

(Chiang et al., 2020; Go et al., 2004; MacRae et al., 2021; Nowakowska et al., 2019; Szekanecz et al., 2019), studies on molecular aspects of co/multimorbidities are lacking before the current study.

Availability of high-throughput biological datasets such as omics and advanced machine learning based data science technologies makes it possible to identify and develop a comprehensive and shared landscape of molecular networks underlying different diseases. Therefore, a promising future direction for co/multimorbidity research is identification of system- and individual-level multiomic biomarkers of known diseases using traditional as well as advanced machine learning approaches, and development of comprehensive database of disease networks of multiomic markers, lifestyle risk factors as well as clinical parameters. The database can play a pivotal role in development of multipurpose prevention and treatment approaches for co/multimorbidity by providing information on key molecules such as genes and lipids that can be targeted for intervention considering their effect on multiple conditions and potential side effects.

7 SUMMARY AND CONCLUSIONS

This study highlights the importance of early identification of people with adverse molecular profiles as risk factors for the two complex diseases, atherosclerosis, and osteoporosis simultaneously. The approach is important as it may allow development of better prevention programs of these co/multimorbidity conditions associated with high healthcare costs worldwide. The present study was designed to identify these novel molecular and system-level multiomic biomarkers of atherosclerosis-osteoporosis co/multimorbidity.

The main conclusions of this thesis are as follows:

1. In the studied participants, a plasma lipidome module containing 105 densely connected lipid species, mostly from classes of glycerolipids, glycerophospholipids and sphingolipids, was associated with early markers of both atherosclerosis and osteoporosis. The lipid species belonging to sphingolipid class involved several ceramide species. These findings confirm the significance of ceramides in cardiovascular diseases and in addition suggest their potential role as joint biomarkers for atherosclerosis and osteoporosis (study I).

2. There were two gene modules associated with early markers of both atherosclerosis and osteoporosis. We pinpointed three most important genes (NOSIP, GXYLT2 and TRIM63) from the two modules that might play important role as biomarkers of the two diseases (study II).

3. In multivariate analysis of variance of human plasma lipidome at molecular level, we uncovered four lipid species, TAG(18:0/18:0/18:1), PC(40:3), Gb3(d18:1/22:0) and Gb3(d18:1/24:0) jointly associated with the early markers of the studied two diseases (study III).

4. In gene set analysis of blood transcriptomics data, we identified three novel biological processes associated with early markers of atherosclerosis but not

with those of osteoporosis. The findings enhance our understanding of biological processes underlying the early phase of atherosclerosis (study IV).

Overall, the findings of this study support atherosclerosis-osteoporosis co/multimorbidity. The lipidomic and transcriptomic biomarkers uncovered in this study may provide new biomarkers for the co/multimorbidity and help in developing dual-purpose prevention and treatment measures for both diseases. Most importantly, this study shifts the focus of current biomedical research from single disease to spectrum of diseases that may co-exist and share common molecular mechanisms. The study also introduces novel bioinformatic approaches to study co/multimorbidity in general.

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9 PUBLICATIONS

PUBLICATION I

Shared lipidomic architecture by subclinical markers of osteoporosis and atherosclerosis: The Cardiovascular Risk in Young Finns Study

Binisha H. Mishra, Pashupati P. Mishra, Nina Mononen, Mika Hilvo, Harri Sievänen, Markus Juonala, Marika Laaksonen, Nina Hutri-Kähönen, Jorma Viikari, Mika Kähönen, Olli T. Raitakari, Reijo Laaksonen, Terho Lehtimäki

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Full Length Article

Lipidomic architecture shared by subclinical markers of osteoporosis and atherosclerosis: The Cardiovascular Risk in Young Finns Study

Binisha H. Mishra[a,b,c,*], Pashupati P. Mishra[a,b,c], Nina Mononen[a,b,c], Mika Hilvo[d], Harri Sievänen[e], Markus Juonala[f,g,h], Marika Laaksonen[i], Nina Hutri-Kähönen[j], Jorma Viikari[f,g], Mika Kähönen[b,k], Olli T. Raitakari[h,l,m], Reijo Laaksonen[a,b,d], Terho Lehtimäki[a,b,c]

[a] Department of Clinical Chemistry, Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland
[b] Finnish Cardiovascular Research Centre, Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland
[c] Department of Clinical Chemistry, Fimlab Laboratories, Tampere, Finland
[d] Zora Biosciences Oy, Espoo, Finland
[e] UKK Institute, Tampere, Finland
[f] Department of Medicine, University of Turku, Turku, Finland
[g] Division of Medicine, Turku University Hospital, Turku, Finland
[h] Research centre of Applied and Preventive Cardiovascular Medicine, University of Turku, Turku, Finland
[i] Fazer Lab Research, Oy Karl Fazer Ab, Helsinki, Finland
[j] Department of Paediatrics, Tampere University Hospital, Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland
[k] Department of Clinical Physiology, Tampere University Hospital, Tampere Finland.
[l] Department of Clinical Physiology and Nuclear Medicine, Turku University Hospital, Turku, Finland
[m] Centre for Population Health Research, University of Turku and Turku University Hospital, Turku Finland

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ABSTRACT

Background: Studies have shown that osteoporosis and atherosclerosis are comorbid conditions sharing common risk factors and pathophysiological mechanisms. Understanding these is crucial in order to develop shared methods for risk stratification, prevention, diagnosis and treatment. The aim of this study was to apply a system-level bioinformatics approach to lipidome-wide data in order to pinpoint the lipidomic architecture jointly associated with surrogate markers of these complex comorbid diseases.

Subjects and methods: The study was based on the Cardiovascular Risk in Young Finns Study cohort from the 2007 follow-up (n = 1494, aged 30–45 years, women: 57%). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to analyse the serum lipidome, involving 437 molecular lipid species. The subclinical osteoporotic markers included indices of bone mineral density and content, measured using peripheral quantitative computer tomography from the distal and shaft sites of both the tibia and the radius. The subclinical atherosclerotic markers included carotid and bulbus intima media thickness measured with high-resolution ultrasound. Weighted co-expression network analysis was performed to identify networks of densely interconnected lipid species (i.e. lipid modules) associated with subclinical markers of both osteoporosis and atherosclerosis. The levels of lipid species (lipid profiles) of each of the lipid modules were summarized by the first principal component termed as module eigenlipid. Then, Pearson's correlation (r) was calculated between the module eigenlipids and the markers. Lipid modules that were significantly and jointly correlated with subclinical markers of both osteoporosis and atherosclerosis were considered to be related to the comorbidities. The hypothesis that the eigenlipids and profiles of the constituent lipid species in the modules have joint effects on the markers was tested with multivariate analysis of variance (MANOVA).

Results: Among twelve studied molecular lipid modules, we identified one module with 105 lipid species significantly and jointly associated with both subclinical markers of both osteoporosis ($r = 0.24$, p-value $= 2 \times 10^{-20}$) and atherosclerosis ($r = 0.16$, p-value $= 2 \times 10^{-10}$). The majority of the lipid species in this module belonged to the glycerolipid (n = 60), glycerophospholipid (n = 13) and sphingolipid (n = 29) classes. The module was also enriched with ceramides (n = 20), confirming their significance in cardiovascular outcomes and suggesting their joint role in the comorbidities. The top three of the 37 statistically significant (adjusted p-value < 0.05) lipid species jointly associated with subclinical markers of both osteoporosis and atherosclerosis within the module were all triacylglycerols (TAGs) – TAG(18:0/18:0/18:1) with an adjusted p-

* Corresponding author at: Department of Clinical Chemistry, Fimlab Laboratories and Finnish Cardiovascular Research Centre Tampere, Finland
E-mail address: binisha.hamalmishra@tuni.fi (B.H. Mishra).

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value of 8.6×10^{-8}, TAG(18:0/18:1/18:1) with an adjusted p-value of 3.7×10^{-6}, and TAG(16:0/18:0/18:1) with an adjusted p-value of 8.5×10^{-6}.

Conclusion: This study identified a novel lipid module associated with both surrogate markers of both subclinical osteoporosis and subclinical atherosclerosis. Alterations in the metabolism of the identified lipid module and, more specifically, the TAG related molecular lipids within the module may provide potential new biomarkers for testing the comorbidities, opening avenues for the emergence of dual-purpose prevention measures.

1. Introduction

Cardiovascular diseases and osteoporosis are both widely prevalent disorders, inducing serious morbidities, bone fractures and death [1–4]. Evidence indicates that there is a similar pathophysiological mechanism underlying both diseases [5]. Several association studies have linked bone measures with atherosclerosis-related measures, such as echogenic calcified atherosclerotic plaques, pulse wave velocity and coronary artery calcification [3,6–8]. Using human atherosclerotic plaque transcriptomics and confocal microscopic analysis, we have shown that advanced atherosclerotic lesions express a variety of markers related with osteoclastogenesis, osteoblastogenesis and calcification and that they involve osteoclast-like cells [9]. Furthermore, genetic polymorphism of apolipoprotein E, a key regulator of serum lipid levels [10] and atherosclerosis [11], has also been shown to be associated with bone structural traits [12]. Various studies have also revealed a positive biological effect of statin, a cholesterol-lowering drug used for the prevention of cardiovascular diseases, to be effective on bone density [13,14]. However, there are also studies that reveal no significant association between the bone and vascular markers [15–17]. Although, they share the same biomarkers and risk factors – for example, oestrogen deficiency, vitamin D abnormalities, dyslipidaemia, smoking, physical inactivity, intake of dietary calcium, dietary saturated fat, oxidative stress and genetic factors [18–21] – the nature and the mechanism involved remains elusive.

Lipidomics offers a tool to investigate the systemic lipid profiles produced in the body's cells, tissues and organs, as well as their interactions with other molecular and cellular components [22]. An altered lipidome has been shown to be associated with several clinical conditions [23,24]. Understanding these alterations can provide useful insight into the development process of the diseases. A study investigating the shared underlining mechanism of atherosclerosis and osteoporosis comorbidity by utilizing lipidomics data is lacking. Therefore, the objective of the present study was to perform a system-level analysis of lipidomics data to identify networks of lipid species associated jointly with subclinical markers of both osteoporosis and atherosclerosis.

The lipidomics data in this study involved 437 molecular lipids generated with liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique from the serum of 1494 participants. Traditional individual molecule-wise statistical methods are limited in their ability to provide a holistic system-level picture. We, therefore, performed a signed weighted lipid co-expression network analysis to identify networks of lipid species (modules) jointly associated with subclinical markers of both osteoporosis and atherosclerosis [25].

2. Material and methods

2.1. Study subjects

The Cardiovascular Risk in Young Finns Study (YFS) is a prospective multi-centre follow-up study investigating cardiovascular risk factors from childhood to adulthood [26]. The study was initiated in 1980 with 3596 children and adolescents aged 3–18 years. The participants were randomly selected from the areas of five university hospitals in Finland (Turku, Tampere, Helsinki, Kuopio and Oulu) and have been followed for nearly 40 years. The present study is based on 1494 participants aged 30–45 from the 2007 follow-up, with four atherosclerotic and six osteoporotic markers, as summarized in Table 2.

2.2. Measurement of surrogate markers of subclinical atherosclerosis

Carotid and bulbus intima-media thickness (IMT) were used as surrogate markers of subclinical atherosclerosis. An ultrasound imaging device with a high-resolution system (Sequoia 512, Acuson) including 13.0 MHz linear array transducers was used for IMT measurement by trained sonographers following a standardized protocol. The image was focused on the posterior (far) wall, and images were recorded from the angle showing the greatest distance between the lumen–intima interface and the media–adventitia interface. A scan including the beginning of the carotid bifurcation and the common carotid artery was recorded and stored in digital format on optical discs for subsequent off-line analysis. All scans were analysed by one reader blinded to the participants' details. The best-quality end-diastolic frame was selected. Several measurements of the common carotid far wall were taken approximately 10 mm proximally to derive the maximal carotid IMT. To assess the reproducibility of the IMT measurements, we re-examined 60 participants 3 months after the initial visit (2.5% random sample). The between-visit coefficient of variation of IMT measurements was 6.4%. To assess the reproducibility of the IMT image analysis, 113 scans were re-analysed by a second observer, and the coefficient of variation was 5.2%. The mean and maximum carotid and bulbus IMT was used in the study.

2.3. Measurement of surrogate markers of subclinical osteoporosis

Two trained researchers in each study centre performed the peripheral quantitative computed tomography (pQCT) bone measurements from both the distal and the diaphysis sites of the radius and tibia. The same pQCT device was used in all five centres (XCT 2000R, Stratec, Medizintechnik, Pforzheim, Germany). The tomographic slices were taken from the shaft (a cortical-rich bone site) and the distal part (a trabecular-rich bone site) of the weight-bearing tibia (30% and 5% from the distal endplate of the tibia, respectively) and of the nonweight-bearing radius (30% and 4% from the distal endplate of the radius, respectively) according to our standard procedures [27]. For the shaft regions, the analysed bone traits were total area (ToA, mm^2), cortical area (CoA, mm^2), and cortical density (CoD, mg/cm^3). For the distal parts of the radius and tibia, the measured bone traits were ToA (mm^2), CoA (mm^2) and trabecular density (TrD, mg/cm^3). The range of in vivo precision of the used pQCT-measured traits ranged from 0.5% (CoD of the radial shaft) to 4.4% (CoA of the distal radius). Mineral content was calculated as $0.2 \times (\text{area}/100) \times \text{density}$. The measured indices are demonstrated in Table 2.

2.4. Health and life style data

The physical activity index was calculated as metabolic equivalents (METs) by combining information on the frequency, intensity and duration of physical activity including leisure-time physical activity and commuting to the workplace (MET h/wk). One MET corresponds to the energy consumption of one kilocalorie per kilogram of weight per hour at rest [28]. Alcohol consumption was measured by asking participants to report their alcohol consumption during the previous week. One unit is equivalent to 14 g of alcohol [29].

2.5. Lipidome-wide analysis

Lipidome quantification for the stored serum samples was performed at Zora Biosciences Oy (Espoo, Finland). Lipid extraction was based on a previously described method [30]. In brief, 10 µl of 10 mM 2,6-di-tert-butyl-4-methylphenol (BHT) in methanol was added to 10 µl of the sample, followed by 20 µl of internal standards (Avanti Polar Lipids Inc., Alabaster, AL) and 300 µl of chloroform:methanol (2:1, v:v) (Sigma-Aldrich GmbH, Steinheim, Germany). The samples were mixed and sonicated in a water bath for 10 min, followed by a 40-min incubation and centrifugation (15 min at 5700 $\times g$). The upper phase was transferred and evaporated under nitrogen. Extracted lipids were resuspended in 100 µl of water-saturated butanol and sonicated in a water bath for 5 min. Then, 100 µl of methanol was added to the samples before the extracts were centrifuged for 5 min at 3500 $\times g$, and finally the supernatants were transferred to the analysis plate for mass spectrometric (MS) analysis. The MS analyses have also been described in detail previously [31]. The analyses were performed on a hybrid triple quadrupole/linear ion trap mass spectrometer (QTRAP 5500, AB Sciex, Concord, Canada) equipped with ultra-high-performance liquid chromatography (UHPLC) (Nexera-X2, Shimadzu, Kyoto, Japan). Chromatographic separation of the lipidomic screening platform was performed on an Acquity BEH C18, 2.1 × 50 mm id. 1.7 µm column (Waters Corporation, Milford, MA, USA). The data were collected using a scheduled multiple reaction monitoring algorithm and processed using Analyst and MultiQuant 3.0 software (AB Sciex). The heights of the peaks obtained from the MS analysis were normalized with the internal standard of the lipid classes.

2.6. Biostatistical analysis

The lipid profiles were $\log_e$ transformed to correct for skewness. We used signed weighted co-expression network analysis implemented in R statistical software [25] to identify groups of densely interconnected lipid species, hereafter referred to as lipid modules. The analysis pipeline is illustrated in Fig. 1. Pearson's correlation coefficients (r) were calculated for all pairwise comparisons of lipid species across all participants. The correlation matrix was transformed to an adjacency matrix by raising it to the power of 5, chosen based on scale-free topology criteria (Fig. S1). The power transformation reduces noise by supressing low correlations and emphasizing stronger correlations between lipid species. The power term is chosen in a manner that leads to

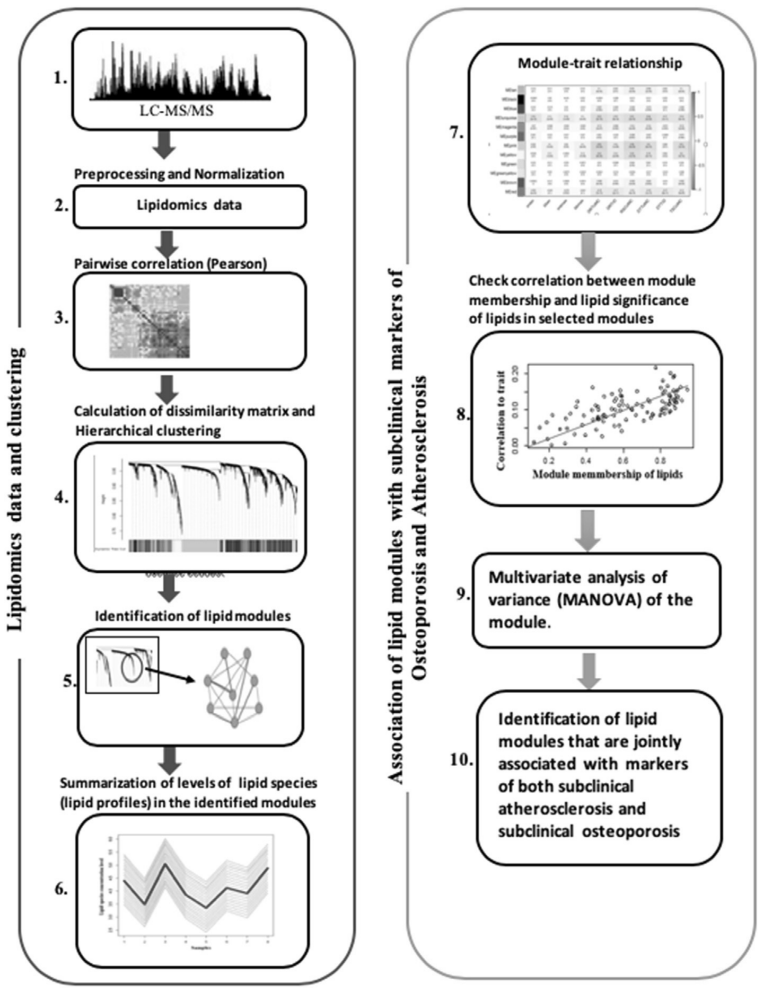

Fig. 1. Weighted co-expression network analysis pipeline. 1) Lipidomics data generated with liquid chromatography-tandem mass spectrometry technique (LC-MS/MS). 2) Levels of lipid species. 3) Correlation matrix based on the pairwise correlation (Pearson) of the lipid species. 4) Hierarchical clustering of the dissimilarity matrix generated from the correlation matrix. 5) Identification of lipid species modules based on clustering. 6) Summarization of the levels of constituent lipid species in the modules by calculating their first principal component called as eigenlipid. 7) Correlation between eigenlipids (as representative of modules) and markers of subclinical osteoporosis and atherosclerosis. 8) Examination of the correlation between module membership and the lipid significance of lipid species in the selected modules as a quality check of the modules.

a scale-free network topology because most of the biological networks are expected to be approximately scale-free. The resulting adjacency matrix was used to generate a Topological Overlap Matrix (TOM). The TOM is a pairwise similarity matrix of the lipid species that considers topological similarity among lipid species. For example, a high TOM implies that a pair of lipid species shares several neighbour lipid species with similar levels. The TOM was transformed into a dissimilarity matrix. Average linkage hierarchical clustering of the dissimilarity matrix was performed to generate a hierarchical clustering tree of lipid species. Lipid modules that are weighted networks of lipid species were identified with a dynamic tree-cutting algorithm. The lipid profiles in each module were summarized by the module eigenlipid (ME), which is defined as the first principal component of the modules' lipid profiles. Association analysis was performed by calculating Pearson's correlation coefficients (r) between the modules and the studied markers. Multivariate analyses of variance (MANOVA) were conducted for significant modules and their constituent member lipid species in order to test the hypothesis that the eigenlipid and profiles of the constituent lipid species in the modules have joint effects on markers of both subclinical osteoporosis and subclinical atherosclerosis. All the multivariate analyses were adjusted for age and sex. All statistical analyses and data processing were performed using the statistical package R version 3.4.3 [32].

3. Results

3.1. Study population characteristics

The characteristics of the study population are shown in Table 1. The disease incidences are based on self-reports [27]. The measured markers of subclinical osteoporosis and atherosclerosis are shown in Table 2.

3.2. Association between surrogate markers of subclinical osteoporosis and atherosclerosis

The surrogate markers of subclinical osteoporosis had a weak but significant (p-value < 0.01) positive correlation with those of subclinical atherosclerosis (Fig. 2).

3.3. Identification of lipid modules

An adjacency matrix was generated from the correlation matrix of the molecular lipid species using a soft-thresholding power of five with the *WGCNA* R package. The threshold was chosen based on a network topology analysis. The network resembled a scale-free graph, with $r^2 > 0.80$, when the correlation matrix was raised to the power of five (Fig. S1). The hierarchical clustering of the TOM dissimilarity matrix defined 12 modules, containing 6–105 highly correlated lipid species (Fig. S2). The lipid modules were named according to colour for downstream analysis, as shown in Fig. 3.

3.4. Module trait relationships and identification of the most significant modules

Pearson's correlation between the module eigenlipids (MEs) and the studied markers was calculated. Three modules (turquoise, pink and yellow) were found to be significantly associated with several of both the osteoporotic and the atherosclerotic markers (Fig. 3). The turquoise module was significantly associated with the carotid-IMT-related variables *imtav* ($r = 0.16$, p-value $= 2 \times 10^{-10}$) and *imtmax* ($r = 0.16$, p-value $= 1 \times 10^{-9}$). The same module was also significantly associated with all of the pQCT bone measurements, the closest association being with *DTToMC* ($r = 0.24$, p-value $= 2 \times 10^{-20}$). The pink and yellow modules were significantly associated with both bulbus IMT variables *bbav* (pink: $r = 0.10$, p-value $= 7 \times 10^{-5}$, yellow: $r = 0.11$, p-value $= 1 \times 10^{-5}$) and *bbmax* (pink: $r = 0.10$, p-value $= 8 \times 10^{-5}$, yellow: $r = 0.12$, p-value $= 7 \times 10^{-6}$). The same modules were also significantly associated with five of the six pQCT-based subclinical osteoporosis indices. The exact lipid content of the most significant turquoise module is listed in Table S1 and explained under Section 3.6.

3.5. Lipid significance (LS) and module membership (MM)

LS is defined as the correlation between the module's member lipids and the study marker. MM is defined as the correlation between the eigenlipid and the other member lipids. An ideal module is the one where LS and MM are highly correlated suggesting that the lipids that are highly correlated with the biological marker of interest are also the important member of the analysed module [25]. Among the three significant modules (Fig. 4), the joint turquoise module has a highly significant correlation between LS and MM with respect to both subclinical atherosclerotic (*imtav*; $r = 0.66$, p-value $= 1.9 \times 10^{-14}$) and subclinical osteoporotic (*DTToMC*; $r = 0.64$, p-value $= 2 \times 10^{-13}$) markers (Fig. 3). The yellow module has a highly significant correlation between LS and MM only with respect to the subclinical atherosclerotic marker (*bbmax*; $r = 0.49$, p-value $= .00013$), whereas the pink module has no significant correlation between LS and MM with respect to any of the studied markers (data not shown).

3.6. Lipid species distribution in the joint turquoise module for subclinical osteoporotic and atherosclerosis markers

There were 105 lipid species in the joint turquoise module for subclinical markers of osteoporosis and atherosclerosis. The majority of the lipid species belonged to the classes of glycerolipid, glycerophospholipid and sphingolipid (Fig. 5A). The glycerolipid class included 19 diacylglycerol and 41 triacylglycerol (TAG) lipid species (Fig. 5B). The glycerophospholipid class had seven phosphatidylcholine lipid species, and the sphingolipid class was enriched with 20 ceramide species (Fig. 5B).

Table 1
Population characteristics of the Cardiovascular Risk in Young Finns Study cohort. Data are expressed as mean ± SD or percentages.

	Men	Women
Number of subjects	646 (43%)	848 (57%)
Age, years	38 ± 5	38 ± 5
Body mass index, kg/m^2	26.5 ± 3.9	25.1 ± 4.7
Total cholesterol (mmol/l)	5.2 ± 0.9	4.9 ± 0.8
LDL cholesterol (mmol/l)	3.3 ± 0.8	3.0 ± 0.7
HDL cholesterol (mmol/l)	1.2 ± 0.3	1.5 ± 0.3
Triglycerides (mmol/l)	1.6 ± 0.9	1.2 ± 0.6
Serum glucose (mmol/l)	5.5 ± 0.6	5.2 ± 0.7
Insulin (IU/l)	9.9 ± 26.3	8.3 ± 8.6
C-reactive protein (mg/l)	1.6 ± 4.7	2.0 ± 3.5
Systolic blood pressure (mmHg)	125.2 13.1	116 ± 13.4
Diastolic blood pressure (mmHg)	78.3 ± 10.9	72.8 ± 10.7
Alcohol consumption, units/day	1.4 ± 1.9	0.6 ± 0.7
Physical activity index (MET h/wk)	20.4 ± 22.2	19.4 ± 20.1
Daily smoking, %	129/641 (20%)	121/843 (14%)
Daily calcium intake (mg)	1371 ± 602	1190 ± 483
Daily vitamin D intake (μg)	8.4 ± 4.5	7.3 ± 3.5
Family risk factor for Coronary Heart Disease (%)	107/646 (16.6%)	140/847 (16.5%)
Participants with osteoporosis (%)	3/641 (0.5%)	8/845 (1%)
Participants with epilepsy (%)	5/624 (0.8%)	7/835 (0.8%)
Participants with Crohn's disease (%)	5/625 (0.8%)	9/836 (1.1%)
Participants with Anorexia (%)	0	8/836 (1%)
Usage of corticosteroids at least once a month (%)	13/624 (2.1%)	54/837 (6.5%)

Table 2
Surrogate markers of both subclinical osteoporosis and subclinical atherosclerosis with their descriptive statistics among the study participants, expressed as mean ± SD.

Description (unit)	Abbreviations	Mean (± SD)
Subclinical atherosclerosis		
Carotid intima-media thickness (average, mm)	*imtav*	0.6 ± 0.1
Carotid intima-media thickness (maximum, mm)	*imtmax*	0.7 ± 0.2
Bulbus intima-media thickness (average, mm)	*bbav*	0.8 ± 0.1
Bulbus intima-media thickness (maximum, mm)	*bbmax*	0.8 ± 0.1
Subclinical osteoporosis		
Total mineral density of the distal radius's trabecular bone (mg/cm^3)	*DRTrD*	224.4 ± 36.1
Total mineral density of the distal tibia's trabecular bone (mg/cm^3)	*DTTrD*	240.3 ± 34.1
Total mineral content of the distal radius (mg)	*DRToMC*	243.6 ± 64.2
Total mineral content in the radial shaft's cortical bone (mg)	*RSCoMC*	214.2 ± 44.9
Total mineral content in the distal tibia (mg)	*DTToMC*	602.1 ± 126.9
Total mineral content in the tibia shaft's cortical bone (mg)	*TSCoMC*	646.4 ± 110.5

3.7. Multivariate analysis of the turquoise module and its constituent lipid species with subclinical markers of osteoporosis and atherosclerosis

In multivariate analysis of variance (adjusted with age and sex), average carotid intima media thickness (*imtav*) for subclinical atherosclerosis and total mineral content in the distal tibia (*DTToMC*) for subclinical osteoporosis were chosen as outcomes because they obtained the maximum correlation and the minimum p-value in a module–trait relationship analysis (Fig. 3). There was a statistically significant joint association between the turquoise eigenlipid and the markers of subclinical osteoporosis and atherosclerosis, $F(2, 1489) = 12.50$, p-value $= 4.1 \times 10^{-6}$, Pillais' Trace = 0.01. The turquoise eigenlipid had a statistically significant positive association with both markers (p-value with *imtav*: 2.7×10^{-6} and p-value with *DTToMC*: 0.03) in separate regression analyses.

Multivariate analysis of all the member lipid species in the turquoise module with *imtav* and *DTToMC* as outcomes, identified 37 lipid species that were jointly associated with the markers, with a Bonferroni-adjusted p-value of < 0.05 (Table S1). The three most significant joint biomarkers of both osteoporosis and atherosclerosis were TAG (18:0/18:0/18:1), TAG (18:0/18:1/18:1) and TAG (16:0/18:0/18:1), with adjusted p-values of 8.6×10^{-8}, 3.7×10^{-6}, and 8.5×10^{-6}, respectively.

In separate regression analyses of each member lipid species and *imtav*, 36 out of the 37 lipid species were found to be positively associated, with a Bonferroni-adjusted p-value of < 0.05 (Table S2). Similarly, regression analyses of each lipid species with *DTToMC* were also performed. All the 37 lipid species that were found to be jointly associated with markers of subclinical osteoporosis and atherosclerosis were positively associated with *DTToMC*; 16 of these were nominally significant (p-value < 0.05), but none of the lipid species reached a Bonferroni-adjusted p-value of 0.05 (Table S3).

4. Discussion

To the best of our knowledge, this is the first lipidome-wide system-level association study investigating the joint lipid architecture of surrogate markers of both subclinical osteoporosis and subclinical atherosclerosis. We performed lipidomics analysis to identify modules of lipid species that are significantly and jointly associated with the markers' of both of the studied comorbidities. We identified a shared module that is significantly associated with subclinical markers of both osteoporosis (pQCT bone measurements) and atherosclerosis

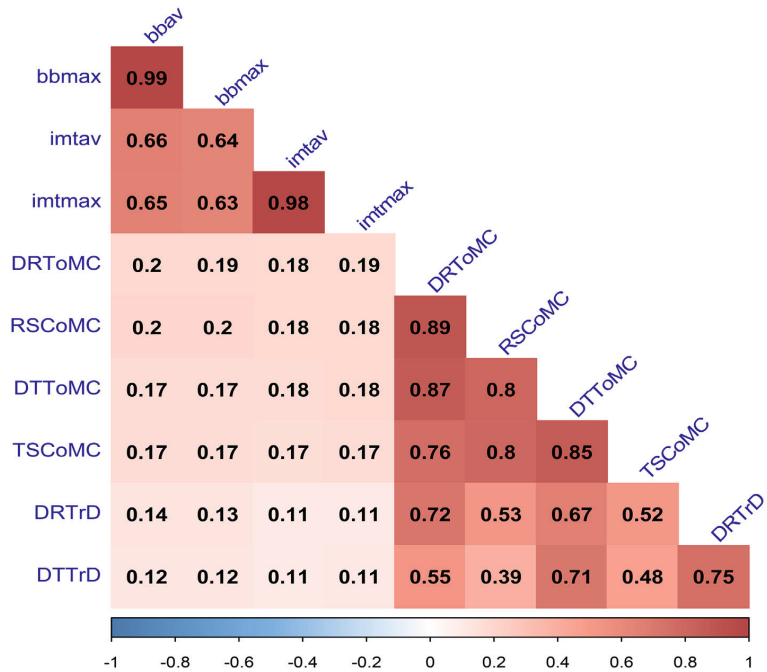

Fig. 2. Pearson's correlation coefficients (r) between surrogate markers of subclinical osteoporosis and atherosclerosis. All correlations are statistically significant (p-value < 0.01). The abbreviations in this figure are explained in Table 2.

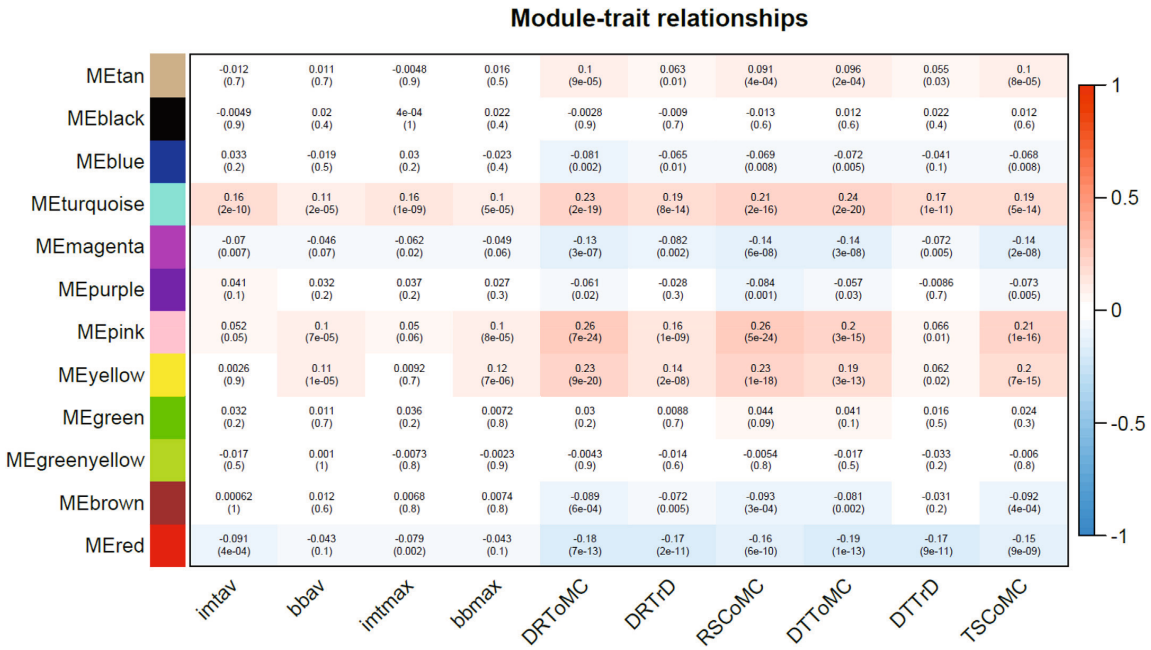

Fig. 3. Module–surrogate marker relationships. The rows correspond to the different modules and their eigenlipids (ME). The columns correspond to the measured subclinical osteoporotic and atherosclerotic markers of the study. The values in the cells represent Pearson's correlation coefficients (r), with the associated p-values in parentheses. The modules are named according to colour and the correlation coefficients have a colour-coding shown in the colour legend (between −1 and +1) on the right side of the figure. The abbreviations for the subclinical osteoporotic and atherosclerotic markers in the column names are explained in Table 2. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

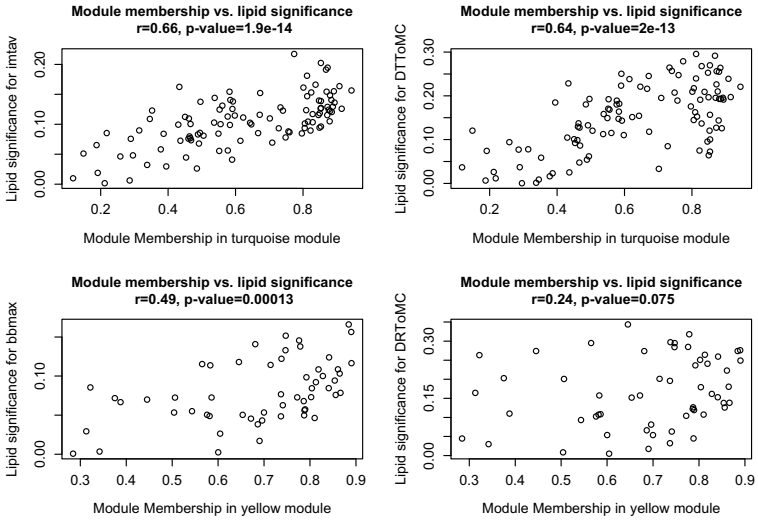

Fig. 4. Scatter plots of lipid significance (LS) vs module membership (MM) in the turquoise and the yellow modules. The left panel corresponds to subclinical atherosclerotic markers and the right panel to subclinical osteoporotic markers. Abbreviations: imtav, carotid intima media thickness (average); bbmax, bulbus intima media thickness (maximum); DTToMC, total mineral content in distal tibia; DRToMC, total mineral content of the distal radius.

(ultrasound carotid IMT).

Whether osteoporosis and atherosclerosis are independent conditions that only share common risk factors, such as aging, or also constitute comorbid conditions with a similar pathophysiological mechanism is an active field of research [19,33]. Several studies have shown an association between decreased bone mass density and increased carotid IMT in different study groups [34–36]. Other studies have suggested an association between osteoporosis and cardiovascular mortality [37–40]. Similarly, one study suggested that defects in bone mineralization and arterial calcification have a similar pathogenesis [41].

In contrast to most of the published findings, we identified weak, but statistically significant positive correlations between surrogate markers of these two diseases. The positive correlations might be due to

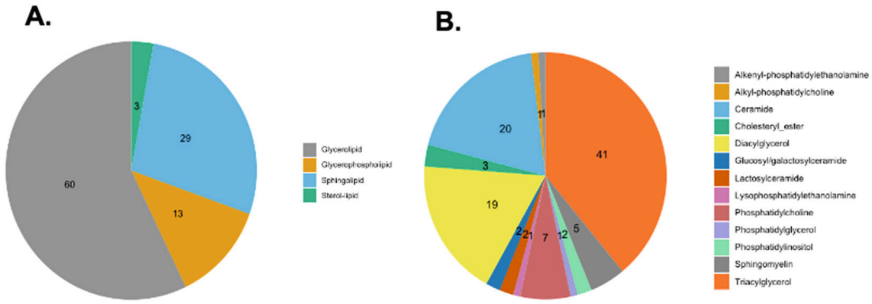

Fig. 5. Distribution of lipids classes (A) and constituent lipids (B) in the joint turquoise module for subclinical markers of osteoporosis and atherosclerosis.

the relatively younger age of the study participants who have not yet developed the clinical manifestations of the diseases. Thus, the positive associations might arise from the shared biological mechanisms between bone and vascular tissue during their normal growth and development. Similar results have been published elsewhere [42]. We speculate that the dynamics of the lipid molecules that are associated with both bone-related and vascular markers change during adverse conditions leading to the comorbidity. Knowledge of the lipid molecules that are associated with the surrogate markers of both the diseases is crucial for identifying alterations in molecular dynamics that take place during the disease. This will not only confirm whether or not the diseases are comorbid but can also potentially improve the risk stratification, prevention, diagnosis and treatment of the diseases.

The majority of the lipid species in the most significant joint module belonged to the glycerolipid, glycerophospholipid and sphingolipid classes. Within glycerophospholipids, one of the phosphatidylcholine lipid species, namely lysophosphatidylcholine (LPC), is a pro-inflammatory lipid that is generated by various pathological activities and is a major component of oxidized low-density lipoprotein (LDL) [43]. Oxidized LDL is known to be a potential factor for the co-occurrence of vascular calcification with the loss of bone mass [44]. Studies have shown that oxidized LDL promotes atherosclerosis via a chemotactic and proliferative mechanism on monocytes by stimulating their adhesion into the endothelial cells and by initiating the formation of foam cells [45]. Oxidized-LDL has also been shown to proliferate and stimulate the migration of smooth muscle cells into the tunica media, which stimulates the production of collagen, thus contributing the fibrous lining in the atherosclerosis plaque [46]. Studies have also suggested that oxidized LDL inhibits osteoblastic differentiation and bone formation and promotes osteoblast cell death [47,48]. A recent study suggested that there is a causal effect of LDL cholesterol on bone mass density [49]. However, clinical findings related to oxidized LDL in the context of cardiovascular diseases have been controversial [50–52].

The identified joint module includes high-risk cardiovascular ceramides among 20 other ceramides, which confirms their previously shown association with cardiovascular outcomes [53,54] and suggests their potential role in subclinical osteoporosis as well. Ceramides are responsible for the activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) that causes the apoptosis of bone cells [55]. A study has demonstrated an association between ceramides and trabecular bone density in mice [56]. In addition, sphingomyelins have been found to be decreased in the bone tissue of mice with osteoporosis [57]. Ceramides also promote lipoprotein infiltration into the vessel wall by acting as a key signalling molecule [58].

An earlier study has shown a significantly increased level of cholesterol ester in the arterial wall of atherosclerotic lesions [59]. An elevated level of triglycerides is known to be an important biomarker in the development of cardiovascular disease [60]. Lipoproteins that carry triglycerides in the blood stream accumulate in the artery wall intima and are taken up by macrophages to form foam cells that contribute to the build-up of plaque along the walls of artery [61]. The triglyceride metabolism in bone tissue has been shown to diminish in subjects with osteoporosis, when compared with the healthy controls [62]. Furthermore, a study with middle-aged women in Japan revealed that patients with hypertriglyceridemia had reduced bone resorption and were at risk of fractures [63]. Furthermore, among the 37 joint lipid species identified herein by multivariate analysis of variance as being significantly associated with both osteoporosis and atherosclerosis, the top three were triglycerides namely TAG(18:0/18:0/18:1), TAG(18:0/18:1/18:1) and TAG(16:0/18:0/18:1). A previous study has shown an association between TAG(18:0/18:1/18:1) and cardiovascular disease [64]. Furthermore, TAG(16:0/18:0/18:1) has been linked to a faster progression of type 2 diabetes [65] which is a risk factor for both cardiovascular disease [66] and bone fractures [67].

This study is limited to the subclinical phase of atherosclerosis and osteoporosis, as it is based on a relatively young cohort population with very few diagnosed cases of cardiovascular disease and osteoporosis. Therefore, further research on lipidome-wide associations with clinical comorbidities in a case–control setting is crucial. Furthermore, as all of the participants of this study are of Caucasian origin, studies with populations of different ethnicities are needed.

5. Summary and conclusion

Several earlier studies have shown that osteoporosis and atherosclerosis are comorbid conditions, emphasizing that these conditions should be investigated in detail to identify common risk factors and joint molecular mechanisms and to develop common methods for risk stratification, prevention, diagnosis and treatment. In the present study, we identified a lipidome module, with its specific molecular lipids, that was significantly associated with surrogate markers of the subclinical phase of both osteoporosis and atherosclerosis. Alteration in the metabolism of the identified lipid species might contribute to the comorbid conditions and yield new possibilities for their dual-based prevention methods.

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Ethical approval

The study was approved by the ethical committee of the Hospital District of Southwest Finland on 20 June 2017 (ETMK:68/1801/2017), and all participants have given an informed written consent. Data protection will be handled according to current regulations.

Declaration of competing interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2019.115160.

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PUBLICATION II

Modular genome-wide gene expression architecture shared by early traits of osteoporosis and atherosclerosis: The Young Finns Study

Binisha H. Mishra, Pashupati P. Mishra, Emma Raitoharju, Saara Marttila, Nina Mononen, Harri Sievänen, Jorma Viikari, Markus Juonala, Marika Laaksonen, Nina Hutri-Kähönen, Mika Kähönen, Olli T. Raitakari, Terho Lehtimäki

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Modular genome-wide gene expression architecture shared by early traits of osteoporosis and atherosclerosis in the Young Finns Study

Binisha H. Mishra[1,2,3]✉, Pashupati P. Mishra[1,2,3], Emma Raitoharju[1,2,3], Saara Marttila[1,2,3,4], Nina Mononen[1,2,3], Harri Sievänen[5], Jorma Viikari[6,7], Markus Juonala[6,7,8], Marika Laaksonen[9], Nina Hutri-Kähönen[10], Mika Kähönen[2,11], Olli T. Raitakari[8,12,13] & Terho Lehtimäki[1,2,3]

We analysed whole blood genome-wide expression data to identify gene co-expression modules shared by early traits of osteoporosis and atherosclerosis. Gene expression was profiled for the Young Finns Study participants. Bone mineral density and content were measured as early traits of osteoporosis. Carotid and bulbus intima media thickness were measured as early traits of atherosclerosis. Joint association of the modules, identified with weighted co-expression analysis, with early traits of the diseases was tested with multivariate analysis. Among the six modules significantly correlated with early traits of both the diseases, two had significant (adjusted p-values (p.adj) < 0.05) and another two had suggestively significant (p.adj < 0.25) joint association with the two diseases after adjusting for age, sex, body mass index, smoking habit, alcohol consumption, and physical activity. The three most significant member genes from the significant modules were NOSIP, GXYLT2, and TRIM63 (p.adj ≤ 0.18). Genes in the modules were enriched with biological processes that have separately been found to be involved in either bone metabolism or atherosclerosis. The gene modules and their most significant member genes identified in this study support the osteoporosis-atherosclerosis comorbidity hypothesis and can provide new joint biomarkers for both diseases and their dual prevention.

Cardiovascular disease and osteoporosis each contribute to a significant disease burden worldwide. It is estimated that roughly 17.9 million people died from cardiovascular disease in 2016, which accounts for 31% of all deaths globally[1]. The estimated number of fragility fractures in the EU in 2010 was 3.5 million, and it is predicted to rise by 28%, reaching 4.5 million, by 2025[2]. Osteoporosis and atherosclerosis share a similar pathophysiological mechanism that involves inflammatory cytokines and oxidized lipids[3]. The two diseases also share several risk factors, such as oestrogen deficiency, vitamin D abnormalities, dyslipidaemia, dietary calcium, dietary saturated fat and oxidative stress, as well as genetic biomarkers, such as osteoprotegerin, apolipoprotein E and the matrix gla protein[3,4]. Plasma lipids such as cholesterol, which has been shown to be associated with atherosclerosis, have

[1]Department of Clinical Chemistry, Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland. [2]Finnish Cardiovascular Research Center Tampere, Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland. [3]Department of Clinical Chemistry, Fimlab Laboratories, Tampere, Finland. [4]Gerontology Research Center (GEREC), Tampere University, Tampere, Finland. [5]The UKK Institute for Health Promotion Research, Tampere, Finland. [6]Department of Medicine, University of Turku, Turku, Finland. [7]Division of Medicine, Turku University Hospital, Turku, Finland. [8]Research Centre of Applied and Preventive Cardiovascular Medicine, University of Turku, Turku, Finland. [9]Fazer Lab Research, Oy Karl Fazer Ab, Helsinki, Finland. [10]Department of Paediatrics, Tampere University Hospital, Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland. [11]Department of Clinical Physiology, Tampere University Hospital, Tampere, Finland. [12]Department of Clinical Physiology and Nuclear Medicine, Turku University Hospital, Turku, Finland. [13]Centre for Population Health Research, University of Turku and Turku University Hospital, Turku, Finland. ✉email: binisha.hamalmishra@tuni.fi

also been shown to be associated with bone health[5,6]. In one of our previous studies, we identified three lipidome-based molecular triglycerides jointly associated with early traits of both osteoporosis and atherosclerosis[7]. Statin, a cholesterol-lowering drug for individuals who are at a high risk of cardiovascular disease, has also been shown to improve bone density[8]. Despite the strong evidence supporting the comorbidity hypothesis, studies investigating the underlying molecular mechanisms of the diseases using omics data, such as transcriptomics, are scarce.

Several transcriptomic studies have been previously carried out on osteoporosis[9,10] and atherosclerosis[11,12] independently. However, multivariate transcriptomics studies investigating the joint association of genes with both osteoporosis and atherosclerosis are lacking. While such multivariate transcriptomics studies are important, the analysis of a large number of genes in such high-dimensional data requires a multitude of tests and, consequently, multiple-testing correction, leading to the exclusion of genes with suggestive significance. However, a system-level bioinformatics approach largely reduces the multiple testing and increases the statistical power. We have recently used such an approach to identify lipidome-based molecular lipids jointly associated with early traits of osteoporosis and atherosclerosis[7].

The objective of the present study was to perform a system-level analysis of genome-wide expression data in whole blood, used as a proxy for difficult-to-acquire samples such as bone or artery wall tissue, to identify gene co-expression modules, enriched pathways in the identified modules and the most important member genes jointly and significantly associated with the early traits of both osteoporosis and atherosclerosis. To achieve the stated goals, we used weighted gene co-expression network analysis (WGCNA)[13], multivariate analysis of variance (MANOVA) and pathway analysis to identify gene modules and enriched pathways jointly associated with early traits of both osteoporosis and atherosclerosis.

Material and methods

Study participants. This study was based on the Young Finns Study (YFS), a prospective multi-centre follow-up study assessing cardiovascular risk factors from childhood to adulthood[14]. The study was initiated in 1980 with 3,596 children and adolescents aged 3–18 years. The participants were randomly selected from the areas of five university hospitals in Finland (Turku, Tampere, Helsinki, Kuopio, and Oulu) and have been followed up for over 40 years. The study was approved by the ethical committee of the Hospital District of Southwest Finland on 20 June 2017 (ETMK:68/1801/2017). All participants gave their written informed consent, and the studies were conducted in accordance with the Declaration of Helsinki. Data protection will be handled according to current regulations. The present study is based on 1,032 participants, aged 30–45 years, from the 2007 follow-up (Table 1), with four atherosclerotic and six osteoporotic traits as summarized in Table 2. The gene expression levels of the study participants were profiled based on the 2011 follow-up.

Assessment of early traits of atherosclerosis. Carotid and bulbus intima-media thickness (IMT) measurements were used as the early traits of atherosclerosis[7]. An ultrasound imaging device with a high-resolution system (Sequoia 512, Acuson) and 13.0 MHz linear array transducers was used for IMT measurements by trained sonographers following a standardized protocol. The image was focused on the posterior (far) wall, and images were recorded from the angle showing the greatest distance between the lumen–intima interface and the media–adventitia interface. A scan including the beginning of the carotid bifurcation and the common carotid artery was recorded and stored in digital format on optical discs for subsequent off-line analysis. All scans were analysed by one reader blinded to the participants' details. The best-quality end-diastolic frame was selected. Several measurements of the common carotid far wall were taken approximately 10 mm proximally to derive the maximal carotid IMT. To assess the reproducibility of IMT measurements, we re-examined 60 participants 3 months after the initial visit (2.5% random sample). The between-visit coefficient of variation of IMT measurements was 6.4%. To assess the reproducibility of the IMT image analysis, 113 scans were re-analysed by a second observer, and the coefficient of variation was 5.2%. The mean and maximum carotid and bulbus IMTs were used in this study.

Assessment of early traits of osteoporosis. The assessment of early traits of osteoporosis was based on peripheral quantitative computed tomography (pQCT) bone measurements from both the distal and diaphysis sites of the radius and tibia, as described elsewhere[7]. The tomographic slices were taken from the shaft (a cortical-rich bone site) and distal part (a trabecular-rich bone site) of the weight-bearing tibia (30% and 5% from the distal endplate of the tibia, respectively), and of the non-weight-bearing radius (30% and 4% from the distal endplate of the radius, respectively) according to our standard procedures[15]. For the shaft regions, the analysed bone traits were total area (ToA, mm^2), cortical area (CoA, mm^2) and cortical density (CoD, mg/cm^3). For the distal parts of the radius and tibia, the measured bone traits were ToA (mm^2), CoA (mm^2) and trabecular density (TrD, mg/cm^3). The in vivo precision of the used pQCT-measured traits ranged from 0.5% (CoD of the radial shaft) to 4.4% (CoA of the distal radius). Mineral content was calculated as 0.2 x (area/100) x density. The measured bone traits are shown in Table 2.

Health and lifestyle data. The physical activity index, calculated as metabolic equivalents (METs) by combining information on the frequency, intensity and duration of physical activity, including leisure-time physical activity and commuting to the workplace (MET h/wk), was used to represent the physical activity of the participants. One MET corresponds to the energy consumption of one kilocalorie per kilogram of weight per one hour at rest[16]. Alcohol consumption was assessed from the participants' reports on their alcohol consumption expressed in units (i.e., 14 g of alcohol) during the previous week[17].

	Men	Women
Number of subjects	454 (44%)	578 (56%)
Age, years	38 (± 5)	38 (± 5)
Body mass index, kg/m^2	26.5(± 4)	25 (± 4.7)
Total cholesterol (mmol/l)	5.2 (± 0.9)	4.9 (± 0.8)
LDL cholesterol (mmol/l)	3.3 (± 0.8)	3.0 (± 0.7)
HDL cholesterol (mmol/l)	1.2 ± (0.3)	1.5 ± (0.3)
Triglycerides (mmol/l)	1.5 ± (0.7)	1.1 (± 0.5)
Serum glucose (mmol/l)	5.4 (± 0.5)	5.2 (± 0.7)
Insulin (IU/l)	10.4 (± 31.2)	8.0 (± 7.6)
C-reactive protein (mg/l)	1.7 (± 5.5)	1.9 (± 3.3)
Systolic blood pressure (mmHg)	124.8 (± 13.3)	116 (± 13.5)
Diastolic blood pressure (mmHg)	78.1 (± 11.1)	72.6 (± 10.9)
Alcohol consumption, units/day	1.4 (± 2.1)	0.6(± 0.7)
Physical activity index (MET-h/wk)	20.5 (± 22.70)	19.1 (± 20)
Daily smoking, %	77/453 (17%)	70/576 (12%)
Daily calcium intake (mg)	1393 (± 613)	1174 (± 453)
Daily vitamin D intake (μg)	8.6 (± 4.6)	7.4 (± 3.4)
Family risk factor for coronary heart disease (%)	72/454 (15.9%)	97/578 (16.8%)
Participants with osteoporosis (%)	3/451 (0.7%)	6/577 (1%)
Participants with epilepsy (%)	3/441 (0.7%)	4/573 (0.7%)
Participants with Crohn's disease (%)	3/442 (0.7%)	5/573 (0.9%)
Participants with anorexia (%)	0	5/573 (0.9%)
Usage of corticosteroids at least once a month (%)	7/442 (2%)	37/573 (6%)
Participants with type 1 diabetes (%)	1/450 (0.2%)	4/578 (0.7%)
Participants with type 2 diabetes (%)	3/449 (0.7%)	3/578 (0.5%)
Participants with menopause (%)	–	0/578 (0%)

Table 1. Population characteristics of the Young Finns Study cohort. Data are expressed as means (± SD) or proportions (%).

Description (unit)	Acronym	Mean ± SD
Early traits of atherosclerosis		
Carotid intima-media thickness (average, mm)	*CIMTavg*	0.63 ± 0.10
Carotid intima-media thickness (maximum, mm)	*CIMTmax*	0.66 ± 0.11
Bulbusintima-media thickness (average, mm)	*BIMTavg*	0.80 ± 0.14
Bulbus intima-media thickness (maximum, mm)	*BIMTmax*	0.83 ± 0.14
Early traits of osteoporosis		
Distal radius trabecular bone mineral density (mg/cm^3)	*DRTrD*	225 ± 36
Distal tibia trabecular bone mineral density (mg/cm^3)	*DTTrD*	241 ± 34
Distal radius total bone mineral content (mg)	*DRToBMC*	245 ± 65
Radial shaft cortical bone mineral content (mg)	*RSCoBMC*	215 ± 45
Distal tibia total bone mineral content (mg)	*DTToBMC*	605 ± 127
Tibia shaft cortical bone mineral content (mg)	*TSCoBMC*	651 ± 110

Table 2. Early traits of osteoporosis and atherosclerosis with their descriptive statistics among the study participants, expressed as mean ± SD.

Blood transcriptomic analysis. RNA isolation was performed from whole-blood samples collected from study participants during the 2011 follow-up. Expression levels were analysed with Illumina HumanHT-12 version 4 Expression BeadChip (Illumina Inc.), containing 47,231 expression and 770 control probes. Samples with fewer than 6,000 significantly detected expression probes (detection p-value < 0.01) were discarded. Raw Illumina summary probe-level data was exported from Beadstudio and processed in R (http://www.r-project.org/) using a nonparametric background correction, followed by quantile normalization with control and expression probes, with the *neqc* function in the limma package[18] and a log2 transformation. Nine samples were excluded due to sex mismatch between the recorded sex and predicted sex based on *RPS4Y1-2* and *XIST* mRNA levels on the Y and X chromosomes, respectively. After quality control, expression data was available for 1,654 samples,

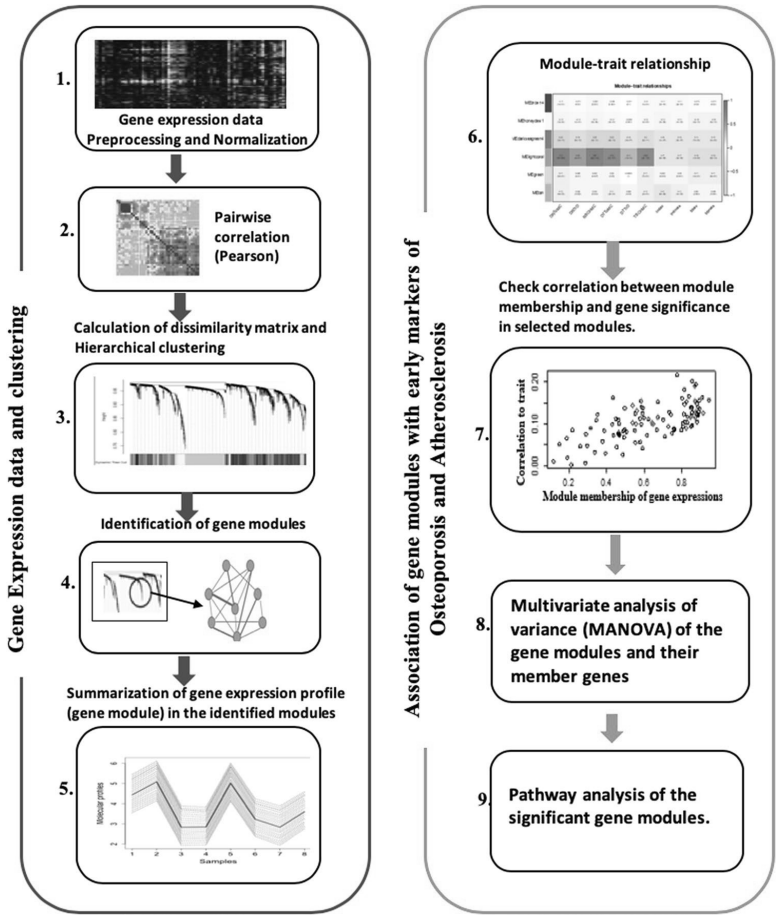

Figure 1. Weighted gene co-expression network analysis pipeline (Figure adapted from Fig. 1 in[7]). 1) Gene expression data analysed using Illumina HumanHT-12 version 4 Expression BeadChip. 2) Correlation matrix based on the pairwise correlations (Pearson) of the gene expression data. 3) Hierarchical clustering of the dissimilarity matrix generated from the correlation matrix. 4) Identification of gene modules based on clustering. 5) Summarization of the gene expression profile in the modules by calculating their first principal component. 6) Correlation between the modules' summary expression profiles (representative of modules) and a set of early traits of osteoporosis and atherosclerosis. 7) Examination of the correlation between module membership and the gene significance of the gene module in the selected modules as a quality check for the modules. 8) Multivariate analysis of variance (MANOVA) of the gene modules and their member genes. 9) Pathway analysis of the member genes of the significant gene modules.

including 4 technical replicates, which were used to examine batch effects and subsequently excluded before further analysis.

Biostatistical analysis. Signed weighted gene co-expression network analysis (WGCNA) implemented with R statistical software[13] was used to identify groups of densely interconnected genes, hereafter referred to as gene modules. The analysis pipeline is illustrated in Fig. 1. The module generation method involved the calculation of Pearson's correlation (r) for all pairwise comparisons of genes across all participants, resulting in a correlation matrix. The correlation matrix was raised to the power of 10 to generate an adjacency matrix in order to minimize noise and emphasize stronger correlations. The power was chosen using the power function implemented in the WGCNA package, to the effect that it transforms the correlation matrix to an approximately scale-free topology based on the assumption that most of the real-world biological networks are scale-free (Figure S1). The resulting adjacency matrix was used to generate a Topological Overlap Matrix (TOM) in order to

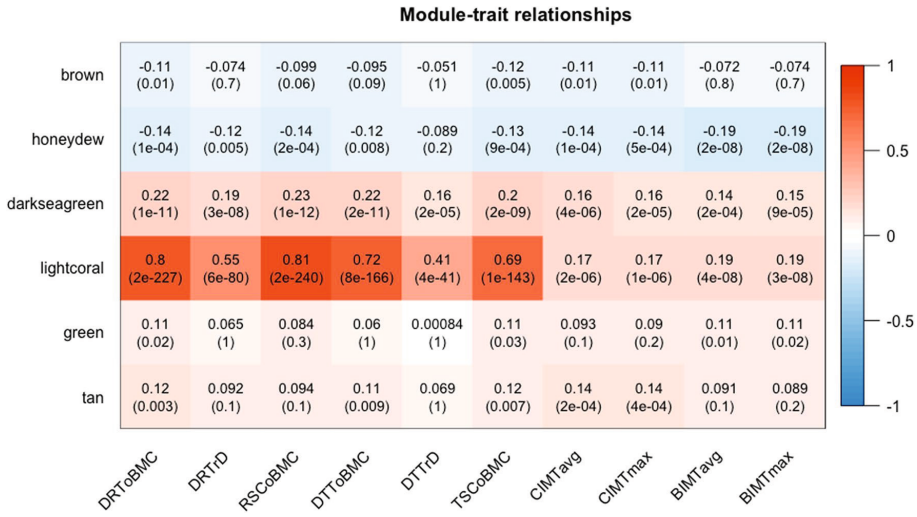

Figure 2. Relationships between gene co-expression modules (x-axis) and early traits (y-axis). The rows correspond to the different gene modules (named by colour) and their summary expression profile; for example, *brown* represents the summary expression profile for the module *brown*. The columns correspond to the measured early traits of osteoporosis (pQCT traits) and atherosclerosis (ultrasound traits) used in the biostatistical analyses. The values in the cells represent Pearson's correlation coefficients (r), with the associated Bonferroni-adjusted p-values in parentheses below the coefficient. The modules are named by colour, and the correlation coefficients have a colour-coding shown in the colour scale (between − 1 and + 1) on the right side of the figure. Acronyms: *DRToBMC*, distal radius total bone mineral content; *DRTrD*, distal radius trabecular bone mineral density; *RSCoBMC*, radial shaft cortical bone mineral content; *DTToBMC*, distal tibia total bone mineral content; *DTTrD*, distal tibia trabecular bone mineral density; *TSCoBMC*, tibia shaft cortical bone mineral content; *CIMTavg*, average carotid intima-media thickness; *CIMTmax*, maximum carotid intima-media thickness; *BIMTavg*, average bulbus intima-media thickness; *BIMTmax*, maximum bulbus intima-media thickness.

incorporate network topology information in the definition of the co-expression of genes. The TOM is a similarity matrix of genes. This was transformed into a dissimilarity matrix, 1-TOM. Average linkage hierarchical clustering of the dissimilarity matrix was performed to generate a hierarchical clustering tree of genes. Next, gene modules were identified with a dynamic tree-cutting algorithm. The quality of the identified gene modules was further assessed by analysing the correlation between gene significance (GS) and module membership (MM). GS is defined as the correlation between the module's member genes and the study traits. MM is defined as the correlation between the summary expression profile of a module and its member genes. An ideal module is one where GS and MM are highly correlated, which suggests that the genes that are highly correlated with the biological marker of interest are also the important member of the analysed module. The first principal component of the expression profiles of the member genes in a module was used as a summary expression profile of the module. Pearson's correlation coefficients (r) were calculated between the summary expression profiles of the identified gene modules and the early traits of osteoporosis and atherosclerosis. Gene modules that were significantly correlated (Bonferroni-adjusted p-value (p.adj) < 0.05) with early traits of both osteoporosis and atherosclerosis were considered as candidate modules for testing joint statistical association with the studied traits of both diseases in multivariate statistical analysis.

A multivariate analysis of variance (MANOVA) test implemented in the *car* R package was performed to test for a joint statistical association between the candidate gene modules and the studied traits of both osteoporosis and atherosclerosis. The MANOVA test was performed with three different models: **model 1** for investigating a joint association between gene modules and early traits of both osteoporosis and atherosclerosis with no covariates; **model 2,** adjusted with age, sex and body mass index (BMI); and **model 3**, which was the same as model 2 but additionally adjusted for smoking, alcohol consumption and variables related to physical activity. The early traits, one for osteoporosis and another for atherosclerosis, that had the most significant correlation with the module's summary expression profile (Fig. 2) were chosen for the test. Biological pathway (groups of biologically related genes) analyses based on the Gene Ontology (GO)[19], the Kyoto Encyclopedia of Genes and Genomes (KEGG)[20] and the Disease Ontology (DO)[21] were performed for the genes involved in the significant gene modules using *clusterProfiler* and *DOSE* R/Bioconductor packages[22,23]. In order to simplify the interpretation, significantly enriched pathways across all of the significant gene modules were clustered using the "*dendextend*" R package based on the similarity of member genes[24].

All statistical analyses and data processing were performed using the statistical package R version 3.4.3[25]. Findings with p.adj < 0.05 were reported as significant. Considering the exploratory nature of this study, p.adj < 0.25 were reported as suggestively significant. Similar reporting approach has been used by others[26].

Results

Study population characteristics. The present study is based on 1,032 participants, aged 30–45 years (56% women), from the Young Finns study 2007 follow-up. The clinical and other detailed characteristics of the study population are shown in Table 1. Number of diseases are based on self-reports[15]. The measured early traits of osteoporosis and atherosclerosis are shown in Table 2. The statistical analyses were based on four atherosclerotic and six osteoporotic traits. The gene expression levels of the study participants were profiled in 2011 follow-up.

Identification of gene modules. The hierarchical clustering of the dissimilarity matrix defined 38 modules, containing 12–5,697 highly correlated genes. Following the standard practice in *WGCNA* analysis, the gene modules were named according to colour for downstream analysis, as shown in Fig. 2.

Gene co-expression module trait relationships and identification of the most significant gene modules. Pearson's correlation coefficients (r) between the modules' summary expression profiles and the studied ten early traits were analysed. Six modules (*brown, honeydew, darkseagreen, lightcoral, green* and *tan*) were found to be significantly correlated with several of the early traits of both osteoporosis and atherosclerosis (Fig. 2). Constituent genes of the six modules are listed in Tables S1–S6. Two modules, *darkseagreen* and *lightcoral*, were significantly correlated with all of the studied early traits of both osteoporosis and atherosclerosis. Within the *lightcoral* module, the correlation values (r) between the module's summary expression profile and different study traits varied between 0.17 and 0.81, and the Bonferroni adjusted p-values (p.adj) for these correlations were between 2×10^{-6} and 2×10^{-240}. Within the *darkseagreen* module, the corresponding r was 0.14–0.23 and p.adj $\leq 2 \times 10^{-4}$ for all traits. Similarly, the module *honeydew* was significantly but inversely correlated with all of the studied traits of atherosclerosis and with all but one (i.e. *DTTrD*) early trait of osteoporosis (r varied between -0.12 and -0.19, p.adj ≤ 0.005 for all traits). The *brown* module was significantly associated with the carotid-IMT-related variables *CIMTavg* (r = -0.11, p.adj = 0.01) and *CIMTmax* (r = -0.11, p.adj = 0.01). The same module was also significantly correlated with three of the pQCT bone measurements, the highest correlation being with *TSCoBMC* (r = -0.12, p.adj = 0.005). Similarly, the *green* and *tan* modules were significantly associated with several early traits of both osteoporosis (r ≥ 0.11, p.adj < 0.05) and atherosclerosis (r ≥ 0.11, p.adj < 0.05). All six significant gene modules (Fig. 2) had a significant correlation between GS and MM with regard to the early traits of both atherosclerosis and osteoporosis, with r ≥ 0.34 and a p-value of ≤ 0.05 (Figure S2–S7).

Multivariate analysis of the six significant gene co-expression modules with early traits of osteoporosis and atherosclerosis. The results from the MANOVA test with the three different models (1–3) described in Sect. 2.6 are presented in Table 3. The Pillai's Trace statistic represents the magnitude of the effect of the module's summary expression profile on the early traits of osteoporosis and atherosclerosis. The value of the statistic ranges from 0 to 1, with higher values meaning higher effect. The F-value represents the predictive ability of a model: a higher F-value suggests higher significance of a model. All six gene modules were jointly and significantly associated with early traits of both osteoporosis and atherosclerosis in model 1 (p.adj $\leq 3.7 \times 10^{-5}$). Two of the six significant modules, *honeydew* and *green*, were jointly and significantly associated with early traits of both osteoporosis and atherosclerosis in models 2 and 3 (p.adj ≤ 0.03).

Multivariate analysis of the member genes of the six significant gene modules with early traits of osteoporosis and atherosclerosis. According to model 1, there were 261 genes significantly and jointly associated with early traits of both osteoporosis (*TSCoBMC*) and atherosclerosis (*CIMTavg*) in the *brown* module (Table S7). The most significant gene (p.adj = 1.2×10^{-10}) in the *brown* module with model 1 is solute carrier family 16 member 10 (*SLC16A10*) (Table 4). Models 2 and 3 identified Zinc Finger Protein 594 (*ZNF594*) as the most significant gene in the *brown* model, with a suggestive significance level (p.adj = 0.08).

According to model 1, the *honeydew* module had 31 genes significantly and jointly associated with early traits of both osteoporosis (*DRToBMC*) and atherosclerosis (*BIMTmax*) (Table S8), the most significant (p.adj = 7.4×10^{-16}) being Nitric Oxide Synthase Interacting Protein (*NOSIP*) (Table 4). *NOSIP* was also the most significant gene of the *honeydew* module with models 2 (p.adj = 0.03) and 3 (p.adj = 0.09).

The *darkseagreen* module had 25 genes significantly and jointly associated with early traits of both osteoporosis (*RSCoBMC*) and atherosclerosis (*CIMTavg*) in model 1 (Table S9), the most significant (p.adj = 6.8×10^{-24}) being Myeloperoxidase (*MPO*) (Table 4). Models 2 and 3 identified Protein Tyrosine Phosphatase Non-Receptor Type 20 (*PTPN20*) as the most significant gene in the *darkseagreen* module, with a p.adj of 0.04 in model 2 and p.adj of 0.13 in model 3.

Only 13 genes in the *lightcoral* module were significantly and jointly associated with early traits of both osteoporosis (*RSCoBMC*) and atherosclerosis (*BIMTmax*) in model 1 (Table S10) and none in models 2 and 3 (Table 4). The most significant gene (p.adj = 1.8×10^{-253}) in model 1, LOC100133662, is uncharacterized.

The *green* module had 18 genes significantly and jointly associated with early traits of both osteoporosis (*DRToBMC*) and atherosclerosis (*BIMTavg*) in model 1 (Table S11), the most significant (p.adj = 3.3×10^{-14}) being MAP7 Domain Containing 2 (*MAP7D2*) (Table 4). Model 2 identified Glucoside Xylosyltransferase 2 (*GXYLT2)* as the most significant gene, with a p.adj of 0.18, and model 3 identified Tripartite Motif Containing 63 (*TRIM63*) as the most significant gene, with a p.adj of 0.07.

Gene modules (number of genes)	Statistical models*	Pillai's Trace	F-value	Bonferroni-adjusted p-value
brown (1,205)	Model 1	0.02	12.18	$\mathbf{5.9 \times 10^{-6}}$
	Model 2	0.003	1.30	0.27
	Model 3	0.002	1.09	0.34
honeydew (33)	Model 1	0.05	26.17	$\mathbf{8.17 \times 10^{-12}}$
	Model 2	0.009	4.0	**0.01**
	Model 3	0.007	4.0	**0.03**
darkseagreen (28)	Model 1	0.07	38.25	$\mathbf{2.2 \times 10^{-16}}$
	Model 2	0.004	2.0	0.13
	Model 3	0.003	2.0	0.19
lightcoral (13)	Model 1	0.66	989.62	$\mathbf{2.2 \times 10^{-16}}$
	Model 2	0.0004	0.23	0.79
	Model 3	0.0004	0.2	0.84
green (3,885)	Model 1	0.02	10.31	$\mathbf{3.7 \times 10^{-5}}$
	Model 2	0.007	4.0	**0.03**
	Model 3	0.008	4.0	**0.02**
Tan (504)	Model 1	0.03	15.44	$\mathbf{2.5 \times 10^{-7}}$
	Model 2	0.004	2.0	0.13
	Model 3	0.003	2.0	0.18

Table 3. Multivariate analysis of variance (MANOVA) test results from association analyses between the summary expression profiles of the six significant gene modules and early traits of both osteoporosis and atherosclerosis, using three differently adjusted models (1–3), as described in the table footnote*. The most significant gene modules across all the three tested models are in bold. Statistical models*: MANOVA model 1 was designed to investigate the joint association between the summary expression profiles of the gene modules and early traits of both osteoporosis and atherosclerosis with no covariates. Model 2 was adjusted with age, sex and body mass index. Model 3 was the same as model 2 but additionally adjusted for smoking, alcohol consumption and variables related to physical activity.

Gene modules	Statistical models*	Genes	Pillai's Trace	F statistics	Bonferroni-adjusted p-value
brown	Model 1	SLC16A10	0.06	31	1.2×10^{-10}
	Model 2	ZNF594	0.02	9.7	0.08
	Model 3	ZNF594	0.02	8.4	0.31
honeydew	Model 1	NOSIP	0.07	40	7.4×10^{-16}
	Model 2	NOSIP	0.01	7	0.03
	Model 3	NOSIP	0.01	6	0.09
darkseagreen	Model 1	MPO	0.1	60	6.8×10^{-24}
	Model 2	PTPN20	0.01	6.6	0.04
	Model 3	PTPN20	0.01	5.4	0.13
lightcoral	Model 1	LOC100133662	0.68	1088	1.8×10^{-253}
	Model 2	EIF1AY	0.0007	3.52	0.39
	Model 3	EIF1AY	0.0006	2.76	0.83
green	Model 1	MAP7D2	0.07	41	3.3×10^{-14}
	Model 2	GXYLT2	0.02	10.1	0.18
	Model 3	TRIM63	0.02	11.1	0.07
tan	Model 1	HS.412918	0.25	169	2.1×10^{-61}
	Model 2	C17ORF28	0.02	7.7	0.23
	Model 3	ESPN	0.01	7.0	0.49

Table 4. Multivariate analysis of variance (MANOVA) test results from association analyses between member genes of the six significant gene modules and early traits of both osteoporosis and atherosclerosis using three differently adjusted models (1–3), as described in the table footnote*. The topmost ranking genes based on the Bonferroni-adjusted p-value for each of the three models are presented. Statistical models*: MANOVA model 1 was designed to investigate joint association between the summary expression profile of the gene modules and early traits of both osteoporosis and atherosclerosis with no covariates. Model 2 was adjusted with age, sex and body mass index. Model 3 was thesame as model 2 but additionally adjusted for smoking, alcohol consumption and variables related to physical activity.

The *tan* module had 132 genes significantly and jointly associated with early traits of both osteoporosis (*DRToBMC*) and atherosclerosis (*CIMTavg*) in model 1 (Table S12), the most significant (p.adj = 2.1×10^{-61}) being an uncharacterized gene (*HS.412918*) (Table 4). Model 2 identified chromosome 17 open reading frame 28 (*C17ORF28*) as the most significant gene in the *tan* module, with a p.adj of 0.23.

Pathway analysis of gene modules shared by early traits of osteoporosis and atherosclerosis. Biological pathways significantly enriched (p.adj < 0.05) across all six gene modules were clustered into four groups based on the similarity of member genes (Fig. 3). The largest cluster, coded with green in Fig. 3, mostly contained pathways related to diseases (including atherosclerosis and mouth disease) and the immune response. The second largest cluster, coded with blue in Fig. 3, contained pathways related to the immune response. The third and fourth clusters (coded with orange and grey, respectively, in Fig. 3) contained pathways respectively related to RNA metabolism and olfactory receptors.

Member genes in the *brown* module were significantly (p.adj < 0.05) enriched with nine GO terms (four biological processes, one molecular function and four cellular components) and two KEGG pathways (Table S13). Eight of the nine pathways belonged to the orange cluster, representing RNA-metabolism-related pathways (Fig. 3). Genes in the *honeydew* module were significantly enriched with 4 GO pathways (2 biological processes and 2 cellular components) (Table S14), all of which were related to the immune response represented by the green cluster (Fig. 3). Genes from the *darkseagreen* module were significantly enriched with 27 GO pathways (17 biological processes, 4 molecular functions and 6 cellular components), 2 KEGG pathways and 15 DO pathways (Table S15). Most of these pathways were related to the immune response and diseases (represented by the green cluster in Fig. 3). The *lightcoral* module contains genes that were significantly enriched with 8 GO pathways (1 biological processes, 3 molecular functions and 4 cellular components), 1 KEGG pathway and 1 DO pathway (Table S16). All the pathways in the *lightcoral* module belonged to the green cluster (Fig. 3). The *green* module was enriched with 6 GO pathways (4 biological processes and 2 molecular functions) and 1 KEGG pathway (Table S17). The pathways are related to olfactory receptors and were represented in the grey cluster (Fig. 3).

Discussion

We performed a system-level analysis of genome-wide gene expression data to identify whole blood transcriptomic modules and the enriched biological pathways shared by early traits of osteoporosis (pQCT bone measurements) and atherosclerosis (ultrasound carotid IMT). Genome-wide expression levels from whole blood represent the average gene expression levels across all cells in whole blood and, therefore, facilitate the examination of the general expression pattern associated with a phenotype being studied. The whole blood gene expression profile recapitulates the biological processes in bone marrow, as immune cells within blood migrate back and forth between blood and bone marrow[27] and are known to influence bone homeostasis[28]. Furthermore, a whole-blood-based approach can provide biomarkers that are easily assessable and non-invasive as opposed to bone and vascular tissue.

We identified six gene modules jointly and significantly associated with the studied traits of both diseases. Two of the six gene modules, *green* and *honeydew*, were jointly and significantly associated with the studied traits of both diseases after adjustment with age, sex, body mass index, smoking habit, alcohol consumption and physical exercise covariates. Another two modules, *darkseagreen* and *tan,* were jointly associated with the studied traits of both diseases after otherwise similar statistical adjustments, but with suggestive significance. A biological pathway analysis of the six gene modules identified several statistically significant biological pathways jointly associated with the studied traits of both diseases. Detailed gene-level analysis of the modules identified the three most significant genes (*NOSIP* from *honeydew1* module, *GXYLT2* and *TRIM63* from *green* module) jointly associated with the early traits of the diseases. The three genes have been identified by previous studies as being independently associated with osteoporosis[29–31] and/or atherosclerosis[32,33], thus validating our findings. However, to the best of our knowledge, this is the first study to report their joint association with early traits of both the diseases.

The biological processes (detection of chemical stimulus, sensory perception of smell and detection of chemical stimulus involved in the sensory perception of smell), molecular function (olfactory receptor activity), and the KEGG (olfactory transduction) pathway enriched in the *green* module mostly contained olfactory receptor genes that belong to the G-protein-coupled receptor gene family. Even though olfactory receptor genes are traditionally known to be responsible for detecting odours and initiating the signalling cascade, various studies have shown their diverse physiological functions in other tissues, such as the testes[34], lungs[35], the brain[36] and the heart[37]. Studies have indicated that olfactory receptors play a critical role in lipid metabolism[38] and regulate heart function[39]. Bone morphogenetic proteins act as a regulator during bone formation and repair[40]. Additionally, bone morphogenetic proteins are suggested to promote the survival of olfactory receptors' neurons[41]. Therefore, we speculate that olfactory receptors have an important role in bone remodelling. The green module was also enriched with a receptor ligand activity (molecular function) pathway that contains genes with anti-inflammatory and anti-atherogenic properties, such as Adiponectin (*ADIPOQ*)[42]. Adiponectin prevents atherosclerosis by decreasing oxidative stress, total cholesterol, triglycerides and low-density lipoprotein-cholesterol[43]. Adiponectin also increases bone mass through the activation of osteoblasts and suppression of osteoclasts[44]. Homophilic cell adhesion via plasma membrane adhesion molecules, a biological process enriched in the green module, contained genes such as the polio virus receptor (*PVR*). *PVR* plays an important role in inflammatory process during atherosclerosis via leukocytes movement across the endothelium[45]. Furthermore, studies indicate that PVR-mediated signalling inhibits osteoclast formation[46].

The biological process, T-cell activation, enriched in the *honeydew* module plays an important role in the development of both osteoporosis[47] and atherosclerosis[48]. Similarly, the other enriched biological process, positive

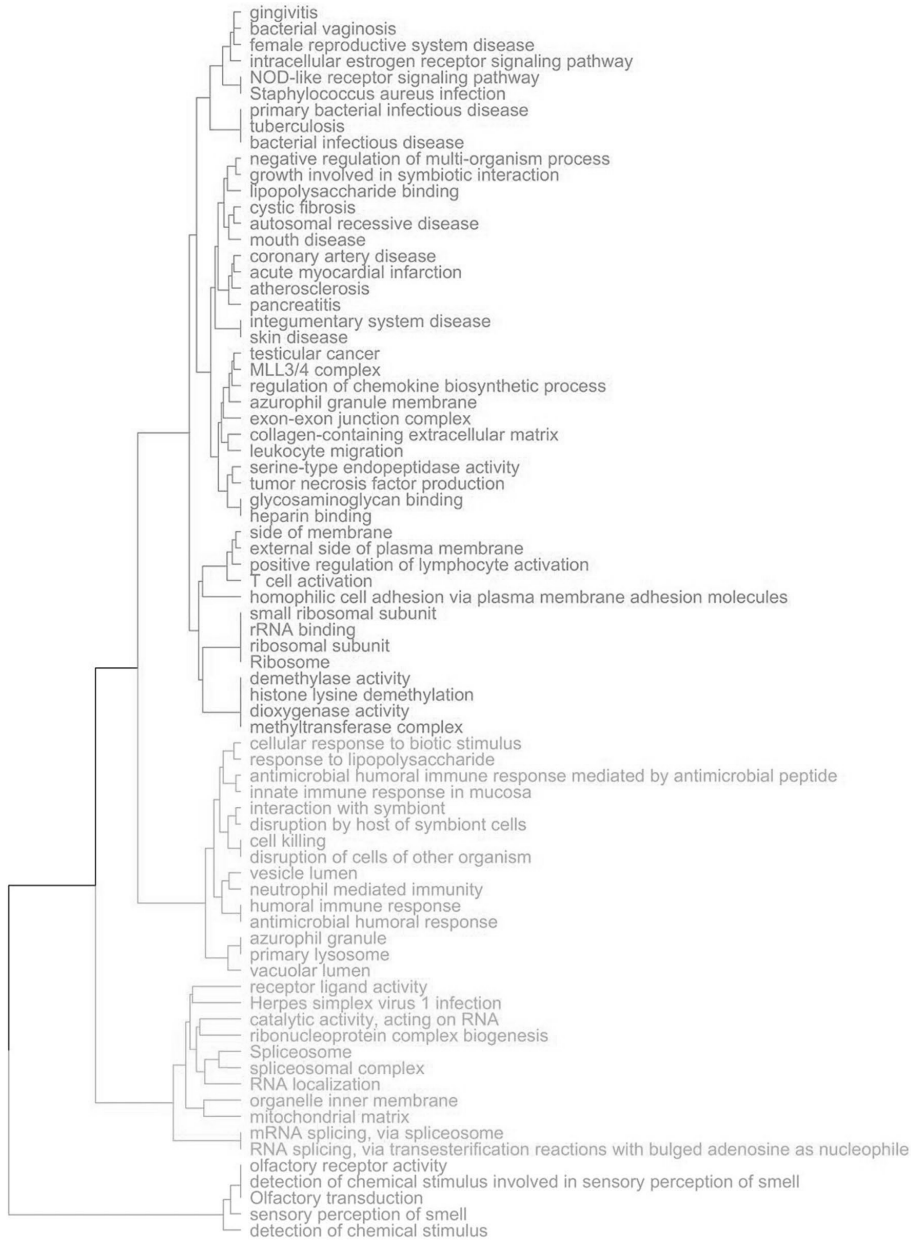

Figure 3. Biological pathways shared by early traits of osteoporosis and atherosclerosis. Dendrogram plot representing the hierarchical clustering of biological pathways significantly enriched (Bonferroni-adjusted p-value < 0.05) in member genes of the six significant joint gene modules (*brown4, honeydew1, darkseagreen4, lightcoral, green* and *tan*). The four clusters, from the largest to smallest in size, are represented by green, blue, orange and grey colours. Biological pathways were defined as sets of genes derived from three different knowledge bases: the Gene Ontology (GO), the Kyoto Encyclopedia of Genes and Genomes (KEGG) and the Disease Ontology (DO).

regulation of lymphocyte activation, is in line with existing literature, as B-lymphocytes are known to regulate the RANK-RANKL-OPG pathway that plays a role in basal bone homeostasis, osteoclast formation and the regulation of bone resorption[49]. Both biological processes are also important for atherosclerosis because the disease involves immune cells such as macrophages and T-lymphocytes[50].

The module *darkseagreen* was enriched with 15 biological pathways based on the Disease Ontology (DO), a knowledge base of human diseases. Three of the DO pathways (coronary artery disease, acute myocardial infarction and atherosclerosis) are related to atherosclerosis, and one of the DO pathways, *mouth disease,* is related to bone disease[51]. There were 17 biological pathways related to biological processes, most of which were related to the immune system, which is central to both bone and vascular health.

The most significant genes jointly associated with early traits of both osteoporosis and atherosclerosis were *NOSIP* (module: *honeydew1*), *GXYLT2* (module: *green)* and *TRIM63* (module: *green*). NOSIP, expressed mostly in musculoskeletal muscle and also in bone-derived and whole blood cells, is related to the metabolism of nitric oxide, which plays a crucial role in the pathogenesis of both osteoporosis[29] and atherosclerosis[32]. A moderate induction of nitric oxide promotes bone resorption, while constitutive production of nitric oxide induces the proliferation of osteoblast-like cells. *GXYLT2* is expressed in whole blood (immune cells) and several other tissues as well. The gene encodes for xylosyltransferase, which plays a role in the biosynthesis of glycosaminoglycan chains, an important constituent of proteoglycans. Proteoglycans are among several extracellular matrix molecules that congregate in atherosclerosis lesions[33] and regulate the osteolytic process[30]. The third significant gene, *TRIM63* (Tripartite Motif Containing 63), also known as *MURF1* (muscle-specific ring finger protein 1), is mostly expressed in skeletal muscle but moderately also in other tissues, such as whole blood (immune cells) and osteoblastic cells, when induced by glucocorticoids. The expression of *TRIM63* has been shown to promote osteoblastic cell differentiation and suppress the proliferation of osteoblastic cells[31]. While *TRIM63* is known to play a role in the regulation of cardiac hypertrophy[52], its role in atherosclerosis is unclear. All three genes are related to the immune system, which plays an important role in the development of both osteoporosis and atherosclerosis. TRIM63 is related to the innate immune system. NOSIP is involved in the metabolism of nitric oxide, which is a key player in the immune system. GXYLT2 plays a role in transferase activity, which also affects immune function.

There were certain limitations to the study. The study was based on a relatively young population cohort with an early phase of cardiovascular disease and osteoporosis and very few clinically diagnosed cases. Therefore, the early traits of the diseases used in the study were positively correlated, as shown in our previous study[7]. A positive correlation between the early traits of osteoporosis and atherosclerosis, such as increasing bone density with an increase in CIMT, is counterintuitive for the comorbidity hypothesis. However, we and others[53] suggest that the positive correlation might be reflecting the shared biological mechanisms between bone and vascular tissue during normal growth and development. The study was thus focused on identifying transcriptomic biomarkers from whole blood that are associated with early traits of bone and vascular health. We speculate that the direction of the association between these two sets of traits representing osteoporotic and atherosclerotic comorbidity and, consequently, also the association with the identified transcriptomic biomarkers, may change systematically due to unhealthy lifestyle choices, similarly to the phenomenon known as "decoherence"[54]. The joint association between the significant modules (*green and honeydew*) and the early traits of the diseases is weak, albeit significant, perhaps due to the relatively young and healthy cohort, as described above. We, however, believe that the results show a suggestive joint association between the modules and traits of the diseases that warrants further research in a case–control setting that includes participants with clinically diagnosed osteoporosis and atherosclerosis. Another limitation of the study was the time difference (four years) between the measurement of the early traits of the diseases and the transcriptomic profile of the study participants. The study was based on the assumption that there is no substantial change in bone and carotid artery measurements over a four-year period among a healthy young population. Also, all study participants are of European origin. Further research in a case–control setting with populations of different ethnicities are needed. This study was based on microarray technology as RNA-Seq technology was still too expensive at the time of follow-up (year 2011) for a large epidemiological study such as the one in this study.

Summary and conclusion

There is a lack of omics-based studies investigating osteoporosis and atherosclerosis comorbidity in the literature despite strong and clear indications from several studies that the diseases are comorbid. We performed system-level analysis of joint associations between early traits of both diseases and transcriptomics modules. The study identified six genome-wide gene co-expression modules, and several enriched biological pathways within the modules, that are significantly and jointly associated with early traits of both the diseases, supporting our comorbidity hypothesis. Detailed analysis of the gene co-expression modules identified three genes (*NOSIP, GXYLT2 and TRIM63*) that might play an important role in developing dual-purpose prevention methods.

Data availability

The dataset supporting the conclusions of this article were obtained from the Cardiovascular Risk in Young Finns study which comprises health related participant data. The use of data is restricted under the regulations on professional secrecy (Act on the Openness of Government Activities, 612/1999) and on sensitive personal data (Personal Data Act, 523/1999, implementing the EU data protection directive 95/46/EC). Due to these restrictions, the data cannot be stored in public repositories or otherwise made publicly available. Data access may be permitted on a case-by-case basis upon request only. Data sharing outside the group is done in collaboration with YFS group and requires a data-sharing agreement. Investigators can submit an expression of interest to

the chairman of the publication committee, Prof Mika Kähönen (Tampere University, Finland) and Prof Terho Lehtimäki (Tampere University, Finland).

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Author contributions

T.L and B.H.M conceptualized the study. B.H.M contributed in data curation, interpretation and writing of the manuscript. P.P.M. adopted the methods for the dataset, performed the statistical analyses and wrote the methodology section. E.R., S.M., N.M., H.S., J.V., M.J., M.L., N.H-.K., M.K., O.R. reviewed and edited the manuscript. T.L and P.P.M supervised the study. All authors read and approved the manuscript.

Competing interests

The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to B.H.M.

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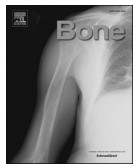

Full Length Article

Uncovering the shared lipidomic markers of subclinical osteoporosis-atherosclerosis comorbidity: The Young Finns Study

Binisha H. Mishra [a,b,c,*], Pashupati P. Mishra [a,b,c], Nina Mononen [a,b,c], Mika Hilvo [d], Harri Sievänen [e], Markus Juonala [f,g], Marika Laaksonen [i], Nina Hutri-Kähönen [j], Jorma Viikari [f,g], Mika Kähönen [b,k], Olli T. Raitakari [h,l,m], Reijo Laaksonen [a,b,d], Terho Lehtimäki [a,b,c]

[a] Department of Clinical Chemistry, Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland
[b] Finnish Cardiovascular Research Centre, Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland
[c] Department of Clinical Chemistry, Fimlab Laboratories, Tampere, Finland
[d] Zora Biosciences Oy, Espoo, Finland
[e] The UKK Institute for Health Promotion Research, Tampere, Finland
[f] Department of Medicine, University of Turku, Turku, Finland
[g] Division of Medicine, Turku University Hospital, Turku, Finland
[h] Research centre of Applied and Preventive Cardiovascular Medicine, University of Turku, Turku, Finland
[i] Fazer Lab Research, Oy Karl Fazer Ab, Helsinki, Finland
[j] Department of Paediatrics, Tampere University Hospital, Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland
[k] Department of Clinical Physiology, Tampere University Hospital, Tampere, Finland
[l] Department of Clinical Physiology and Nuclear Medicine, Turku University Hospital, Turku, Finland
[m] Centre for Population Health Research, University of Turku and Turku University Hospital, Turku, Finland

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ABSTRACT

Background: Osteoporosis and atherosclerosis are complex multifactorial diseases sharing common risk factors and pathophysiological mechanisms suggesting that these are comorbidities. Omics studies identifying joint molecular markers associated with these diseases are sparse.

Subjects and methods: Using liquid chromatography-tandem mass spectrometry, we quantified 437 molecular lipid species from the Young Finns Study cohort (aged 30–45 years and 57% women) and performed lipidome-wide multivariate analysis of variance (MANOVA) with early markers for both diseases. Carotid intima-media thickness for atherosclerosis measured with ultrasound and bone mineral density from distal radius and tibia for osteoporosis measured with peripheral quantitative computed tomography were used as early markers of the diseases.

Results: MANOVA adjusted with age, sex and body mass index, identified eight statistically significant (adjusted *p*-value (p_{adj}) < 0.05) and 15 suggestively significant (p_{adj} < 0.25) molecular lipid species associated with the studied markers. Similar analysis adjusted additionally for smoking habit, physical activity and alcohol consumption identified four significant and six suggestively significant molecular lipid species. These most significant lipid classes/species jointly associated with the studied markers were glycerolipid/*TAG(18:0/18:0/18:1)*, glycerophospholipid/*PC(40:3)*, sphingolipid/*Gb3(d18:1/22:0)*, and sphingolipid/*Gb3(d18:1/24:0)*.

Conclusion: Our results support the osteoporosis-atherosclerosis comorbidity hypothesis and present potential new joint lipid biomarkers for these diseases.

1. Introduction

Osteoporosis and atherosclerosis are both complex multifactorial diseases contributing to significant disease burden worldwide with serious morbidities and death [1,2]. The diseases were considered independent conditions sharing common risk factors such as ageing,

* Corresponding author at: Faculty of Medicine and Health Technology, Tampere University, Tampere 33520, Finland.
E-mail address: binisha.hamalmishra@tuni.fi (B.H. Mishra).

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smoking habit, low physical activity and alcohol intake [3]. However, several studies have identified common pathophysiological mechanisms involving inflammatory cytokines, lipid oxidation products, vitamin D and K deficiency [4–6].

The diseases also share molecular pathways involving bone and vascular mineralization and estrogen deficiency [5,6]. Similarly, *osteoprotegerin*, *matrix-gla protein*, and *apolipoprotein E* have been associated with both atherosclerosis and bone loss [3]. Hence, there is indication of common cellular and molecular process involved in the pathogenesis of both the diseases. Therefore, in-depth understanding of common underlying molecular mechanisms is essential for joint prevention and therapeutics of the diseases.

Lipids play important role in human health and disease. Consequently, dyslipidemia is associated with wide range of diseases [7]. Abnormalities in lipids are risk factors for both osteoporosis [8] and atherosclerosis [9]. For example, elevated total serum cholesterol, triglycerides and low-density lipoprotein (LDL) cholesterol are known risk factors for atherosclerosis [10]. Elevated level of cholesterol [11] and oxidised LDL [12] inhibit osteoblastic differentiation. Statins, cholesterol-lowering drugs for individuals who are at a high risk of cardiovascular disease, have potent positive effects on bone formation [13]. Therefore, biological and epidemiological evidence on dyslipidaemia support the notion that osteoporosis and atherosclerosis are comorbid diseases. However, most of the existing literature is focussed on large lipid classes such as triglycerides, cholesterol and free fatty acids. Deeper insights in lipids and their role in the comorbidities require understanding of dyslipidemia at molecular species level because different molecular weight and lipid component composition might have different biological effects [14,15].

Lipidomics involves identification of the entire spectrum of cellular lipids (lipidome) in biological systems. Lipidome-wide association study of a disease phenotype can identify marker lipid species of the disease that can potentially explain its developmental process. In our recent study, we investigated entire spectrum of lipidomics data to identify networks of lipid modules associated jointly with early markers of both osteoporosis and atherosclerosis [16]. The study was based on modular system-level approach where lipid species were first clustered based on their co-abundance and the statistical analysis was done with the clusters also called as modules. While such system-level approach provides more statistical power for identifying novel lipidomic modules and hub lipid markers within the modules, it can potentially miss to identify individual lipid species that do not cluster well with other lipid species perhaps due to their independent biological role in osteoporosis-atherosclerosis comorbidity. Therefore, in this complementary study, we aim to investigate osteoporosis-atherosclerosis comorbidity hypothesis by performing lipidome-wide multivariate association analysis of early markers of both the diseases to identify molecular lipid species with potentially distinct biological role in the comorbidity.

2. Materials and methods

2.1. Study population

The Cardiovascular Risk in Young Finns Study (YFS) is a Finnish prospective multi-centre longitudinal study investigating cardiovascular risk factors from childhood to adulthood [17]. The study was initiated in 1980 with 3596 children and adolescents aged 3–18 years. The participants were randomly selected from the areas of five university hospital catchment areas in Finland and have been followed for over 40 years. Out of 2200 participants who attended the 27-year follow-up in 2007, we included those for whom measurements of early markers of both osteoporosis and atherosclerosis as well as lipidomic data were available. The present study is, thus, based on 1545 participants (57% were females), aged 30–45 years from the 2007 follow-up, with one atherosclerotic and two osteoporotic early markers, as summarized in Table 1. The YFS was approved by the 1st ethical committee of the Hospital District of Southwest Finland and by local ethical committees (1st Ethical Committee of the Hospital District of Southwest Finland, Regional Ethics Committee of the Expert Responsibility area of Tampere University Hospital, Helsinki University Hospital Ethical Committee of Medicine, The Research Ethics Committee of the Northern Savo Hospital District and Ethics Committee of the Northern Ostrobothnia Hospital District). The study protocol of each study phase corresponded to the proposal by the World Health Organization. All subjects gave written informed consent, and the study was conducted in accordance with the Helsinki declaration. At prior YFS follow-ups, informed consent of every participant under the age of 18 years was obtained from a parent and/or legal guardian.

Table 1
Multivariate analysis of variance (MANOVA) models analyzed in the study.

MANOVA model 1	$(CIMT + DTTrD) \sim lipid$ $(CIMT + DRTrD) \sim lipid$
MANOVA model 2	$(CIMT + DTTrD) \sim lipid + age + sex + body\ mass\ index$ $(CIMT + DRTrD) \sim lipid + age + sex + body\ mass\ index$
MANOVA model 3	$(CIMT + DTTrD) \sim lipid + age + sex + body\ mass\ index + smoking + alcohol\ consumption + physical\ activity$ $(CIMT + DRTrD) \sim lipid + age + sex + body\ mass\ index + smoking + alcohol\ consumption + physical\ activity$

2.2. Measurement of early markers of osteoporosis

Trabecular bone densities (TrD, mg/cm^3) of tibia and radius were used as early markers of osteoporosis. Peripheral quantitative computed tomography (pQCT) was performed at the distal and the diaphysis sites of the radius and tibia. The same pQCT device was used in all five centres (XCT 2000R, Stratec, Medizintechnik GmbH, Pforzheim, Germany). The study is based on tomographic slices taken from the distal part (a trabecular-rich bone site) of the weight-bearing tibia (30% and 5% from the distal endplate of the tibia, respectively) and of the nonweight-bearing radius (30% and 4% from the distal endplate of the radius, respectively) according to standard procedures [18]. Precision of the pQCT methods in this multicentre study was evaluated by performing repeated scans of volunteers in each centre before starting and after completing the measurements. Radius and tibia were measured among 39 women and men twice with repositioning. Reproducibility (coefficient of variation, CV%) was 0.5% for distal tibia TrD and 1.6% for distal radius TrD [18].

2.3. Measurement of early markers of atherosclerosis

Carotid intima-media thickness (CIMT) was used as early marker of atherosclerosis. An ultrasound imaging device with a high-resolution system (Sequoia 512, Acuson) including 13.0 MHz linear array transducers was used for CIMT measurement by trained sonographers following a standardized protocol. The image was focused on the posterior (far) wall, and images were recorded from the angle showing the greatest distance between the lumen–intima interface and the media–adventitia interface. A scan including the beginning of the carotid bifurcation and the common carotid artery was recorded and stored in digital format on optical discs for subsequent off-line analysis. All scans were analyzed by one reader blinded to the participants' details. The best-quality end-diastolic frame was selected. Several measurements of the common carotid far wall were taken approximately 10 mm proximally and mean CIMT was used as the outcome. For reproducibility of the CIMT measurements, we re-examined 60 participants 3 months after the initial visit (2.5% random sample). The between-visit CV% of CIMT measurements was 6.4%. For reproducibility of the CIMT image analysis, 113 scans were re-analyzed by a second observer; CV% was 5.2%.

2.4. Health and lifestyle data

Weekly metabolic equivalent hours (MET-h/wk) calculated from information on the frequency, intensity and duration of physical activity including leisure-time physical activity and commuting to the workplace were used as an index for physical activity. One MET corresponds to the energy consumption of one kilocalorie per kilogram of weight per hour at rest [19]. Information on alcohol consumption was based on participants self-report on their alcohol consumption during the previous week where one unit is equivalent to 14 g of alcohol [20].

2.5. Plasma lipidomic profiling

Lipidome quantification for the stored plasma samples was performed at Zora Biosciences Oy (Espoo, Finland). Lipid extraction was based on a previously described method [21]. In brief, 10 μl of 10 mM 2,6-di-tert-butyl-4-methylphenol (BHT) in methanol was added to 10 μl of sample, followed by 20 μl of internal standards (Avanti Polar Lipids Inc., Alabaster, AL) and 300 μl of chloroform:methanol (2:1, v:v) (Sigma-Aldrich GmbH, Steinheim, Germany). Samples were mixed and sonicated in a water bath for 10 min, followed by a 40-min incubation and centrifugation (15 min at 5700 $\times g$). The upper phase was transferred and evaporated under nitrogen. Extracted lipids were resuspended in 100 μl of water saturated butanol and sonicated in a water bath for 5 min. 100 μl of methanol was added to the samples before the extracts were centrifuged for 5 min at 3500 $\times g$, and finally the supernatants were transferred to the analysis plate for mass spectrometric (MS) analysis. Details of MS analyses have also been described in detail previously [22]. The analyses were performed on a hybrid triple quadrupole/linear ion trap mass spectrometer (QTRAP 5500, AB Sciex, Concords, Canada) equipped with ultra-high-performance liquid chromatography (UHPLC) (Nexera-X2, Shimadzu, Kyoto, Japan). Chromatographic separation of the lipidomic screening platform was performed on Acquity BEH C18, 2.1 × 50 mm id. 1.7 μm column (Waters Corporation, Milford, MA, USA). The data were collected using a scheduled multiple reaction monitoring algorithm and the data were processed using Analyst and MultiQuant 3.0 software (AB Sciex). The heights of the peaks obtained from the MS analysis were normalized with the internal standard of the lipid classes.

2.6. Biostatistical analysis

All the statistical analyses were performed using the R environment for statistical computing, version 3.6.1 [23]. Skewness in the values for body mass index (BMI), physical activity, alcohol consumption and lipidome data were corrected with log2 transformation. Multivariate analyses of variance (MANOVA) with early markers of osteoporosis and atherosclerosis as outcomes and lipidome as predictors were performed using *car* R package [24]. Analyses were done without any covariates (MANOVA model 1) and for two different sets of covariates: one adjusted for age, sex, BMI (MANOVA model 2) and the other adjusted for three additional covariates: smoking habit, physical activity and alcohol consumption (MANOVA model 3). However, as lipidome is known to be affected by age, sex and BMI [25,26], we focus on the results from MANOVA models 2 and 3 in the main manuscript and present results from MANOVA model 1 as Supplementary material. Under each of the MANOVA models, different outcome combinations, for one atherosclerotic and two osteoporosis early markers were analyzed (Table 1). The analysis was also done separately for males and females. In addition, association analysis of lipidome with early markers of osteoporosis and atherosclerosis separately was done with linear regression analyses. For multiple testing correction, appropriate number of independent tests was estimated using the eigenvalues of the correlation matrix using the Matrix Spectral Decomposition (matSpDlite) software [27]. Adjusted *p*-values (p_{adj}) were calculated with Bonferroni method. We report lipid species with $p_{adj} < 0.05$ as significant. Considering the exploratory nature of this study, we also report those lipid species with $p_{adj} < 0.25$ as suggestively significant [28].

3. Results

3.1. Study population characteristics

Population characteristics and measured early markers of osteoporosis and atherosclerosis of the study are shown in Table 2. Number of diseases are based on self-reports [18].

3.2. MANOVA and joint associations of lipid species with early markers of osteoporosis and atherosclerosis

MANOVA model 2 adjusted for age, sex and BMI identified 8 significant ($p_{adj} < 0.05$) and 15 suggestively significant ($p_{adj} < 0.25$) lipid species belonging to four different lipid classes that are jointly associated with the early markers of both diseases (Fig. 1, Tables S11, S14). The four different classes are glycerolipid, glycerophospholipid, sphingolipid and fatty acyl. Glycerolipids include acylglycerols, glycerophospholipids contain a phosphate group esterified to one of the glycerol hydroxyl groups, sphingolipids contain long-chain nitrogenous base and fatty acyls are the most fundamental category as they are building block of complex lipids [29]. As expected, lipids within a class are more correlated among each other than with lipids from other classes (Fig. S1). Among both significant and suggestively significant lipid species, 18 were associated with CIMT and DTTrD and the remaining 5 were associated with CIMT and DRTrD. Only two lipid species *TAG(18:0/18:0/18:1)* and *SM(42:3)* were common for both of

Table 2
Population characteristics and measured early markers of osteoporosis and atherosclerosis of the Young Finns Study cohort. Data are mean (± Standard Deviation) or proportions (%).

	Men	Women
Number of subjects	669 (43%)	876 (57%)
Age, years	38 (±5)	38 (±5)
Body mass index, kg/m^2	27 (±4)	25 (±5)
Total cholesterol (mmol/l)	5.2 (±0.9)	4.9 (±0.8)
LDL cholesterol (mmol/l)	3.3 (±0.8)	3.0 (±0.7)
HDL cholesterol (mmol/l)	1.2 ± (0.3)	1.4 ± (0.3)
Triglycerides (mmol/l)	1.5 ± (0.7)	1.2 (±0.6)
Serum glucose (mmol/l)	5.5 (±0.7)	5.2 (±0.7)
Insulin (IU/l)	10 (±26)	8.5 (±8.5)
C-reactive protein (mg/l)	1.7 (±4.7)	2 (±3.4)
Systolic blood pressure (mmHg)	125 (± 13)	116 (±14)
Diastolic blood pressure (mmHg)	78 (±11)	73 (±11)
Participants with hypertension (%)	40/662 (6%)	45/876 (5%)
Alcohol consumption, units/day	1.4 (±2)	0.6 (±0.7)
Physical activity index (MET-h/wk)	20 (±20)	20 (±22)
Daily smoking, %	129/669 (19%)	119/876 (14%)
Daily calcium intake (mg)	1364 (±606)	1188 (±504)
Daily vitamin D intake (μg)	8.4 (±4.6)	7.4 (±3.6)
Family risk factor for coronary heart disease (%)	109/669 (16%)	156/876 (18%)
Participants with osteoporosis (%)	3/664 (0.5%)	7/874 (0.8%)
Participants with bone fractures (%)	297/647 (46%)	280/866 (32%)
Participants with family history for osteoporosis (%)	25/642 (4%)	61/858 (7%)
Usage of corticosteroids at least once a month (%)	16/647 (3%)	61/865 (7%)
Carotid intima-media thickness (CIMT) (average, mm)	0.65 (±0.11)	0.61 (±0.09)
Participants with CIMT >1 mm	5/669 (0.7%)	0/876
Distal radius trabecular bone mineral density (DRTrD) (mg/cm^3)	247 (±31)	207 (±28)
Distal tibia trabecular bone mineral density (DTTrD) (mg/cm^3)	255 (±33)	229 (±30)

these two early marker combinations shown in Fig. 1. While the most significantly associated lipid species with CIMT and DTTrD was *TAG (18:0/18:0/18:1)*, for CIMT and DRTrD, the most significantly associated species was *Gb3(d18:1/22:0)*. Similar association analyses with MANOVA model 3 (with a stricter model) adjusted with three additional covariates, smoking habit, physical activity and alcohol consumption, identified 4 significant ($p_{adj} < 0.05$) and 6 suggestively significant ($p_{adj} < 0.25$) lipid species belonging to three different lipid classes that were jointly associated with the early markers of both the diseases (Fig. 2, Tables S12, S15). All the 10 lipid species were also present in the results from MANOVA model 2. MANOVA analyses with model 1 with CIMT-DRTrD and CIMT-DTTrA as outcomes identified 202 and 154 significant ($p_{adj} < 0.05$) lipid species respectively (Tables S10, S13).

3.3. MANOVA and sex-specific joint associations of lipid species with early markers of osteoporosis and atherosclerosis

Sex-stratified associations between lipid species and early markers of osteoporosis and atherosclerosis were investigated with MANOVA model adjusted with age, BMI, smoking habit, physical activity and alcohol consumption. *TAG(18:0/18:0/18:1)* and *PC(39:6)* were identified to be female specific and *SM(42:3)* and *SM(44:3)* were identified to be male specific with suggestive statistical significance threshold ($p_{adj} < 0.25$). Note that these lipid species were also found in general analysis above.

3.4. Associations of lipid species with early markers of osteoporosis and atherosclerosis separately

Associations between lipid species and early markers of atherosclerosis (CIMT) or osteoporosis (DTTrD, DRTrD) separately were analyzed with linear regression models similar to that of MANOVA models except that only one marker (either for osteoporosis or atherosclerosis) was used as outcome in each model (Table 1). There were 130 lipid species associated with CIMT with model 1 (without any covariates) with p.adj < 0.25 (Table S1). However, only three (*TAG(18:0/18:0/18:1)*, *AcylCarnitine(16:1) and SM(31:2)*) remained significant after adjusting for age, sex and BMI with model 2 (Table S2) and two (*TAG(18:0/18:0/18:1)*, *AcylCarnitine(16:1)*) remained significant after adjusting additionally for alcohol consumption, smoking and physical activity in model 3 (Table S3). Similarly, 142 and 200 lipid species were associated with early markers of osteoporosis, DTTrD and DRTrD respectively with model 1 (Tables S4, S7). However, with model 3 only four lipid species (*PC(40:3), PC(36:7), PC(36:1), PC(38:3)*) were associated with DTTrD (Table S6) and only three (*Gb3(d18:1/22:0)*, *Gb3 (d18:1/24:0)*, *PC(40:3)*) with DRTrD (Table S9). These findings are similar to those obtained from MANOVA with the osteoporosis and atherosclerosis related outcomes combined as described in Section 3.2.

4. Discussion

To the best of our knowledge, this is the first lipidome-wide association study investigating the joint associations between molecular lipid species and early markers of both osteoporosis and atherosclerosis. Using lipidomics data, we identified molecular lipid species that are jointly associated with early markers of both the studied diseases. We identified eight plasma molecular lipid species jointly and significantly associated with the studied early markers of osteoporosis (trabecular bone density determined with pQCT) and atherosclerosis (carotid intima-media thickness determined with ultrasound).

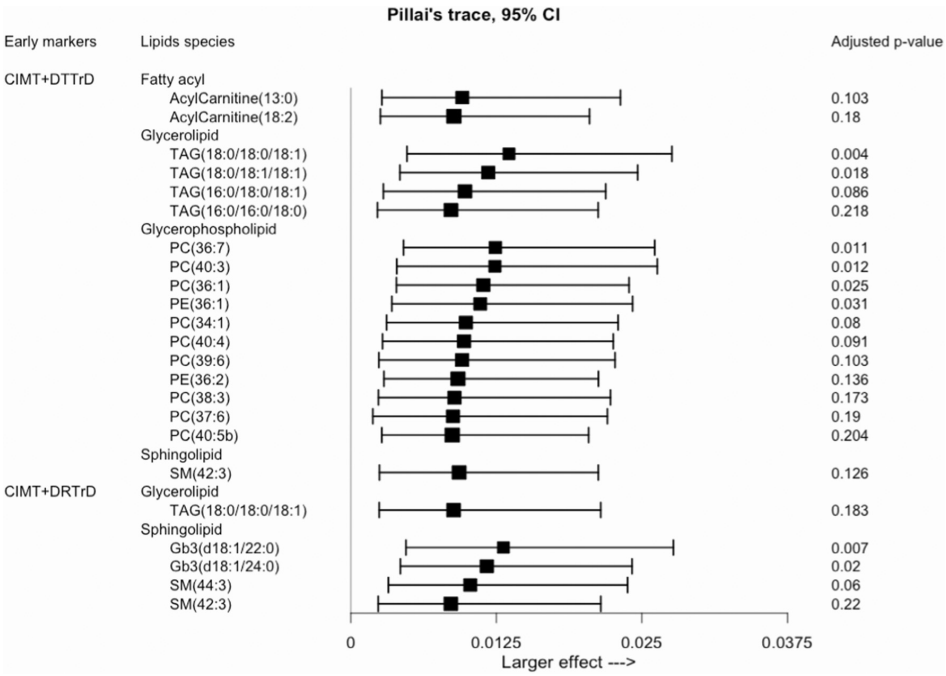

Fig. 1. Forest plot of the multivariate lipidome-wide association study of early markers of osteoporosis and atherosclerosis adjusted for age, sex and body mass index. Analysis was performed for two different outcomes based on combination of early markers of atherosclerosis, carotid intima media thickness (CIMT); and osteoporosis, distal tibia's trabecular bone (DTTrD) and distal radius's trabecular bone (DRTrD). The second column represents lipid species and the classes they belong to. Pillai's trace is the test statistic in multivariate analysis of variance (MANOVA) ranging from 0 to 1. Confidence interval of the test statistic was calculated from 100 bootstraps of the original data. *P*-values were adjusted using Bonferroni's method.

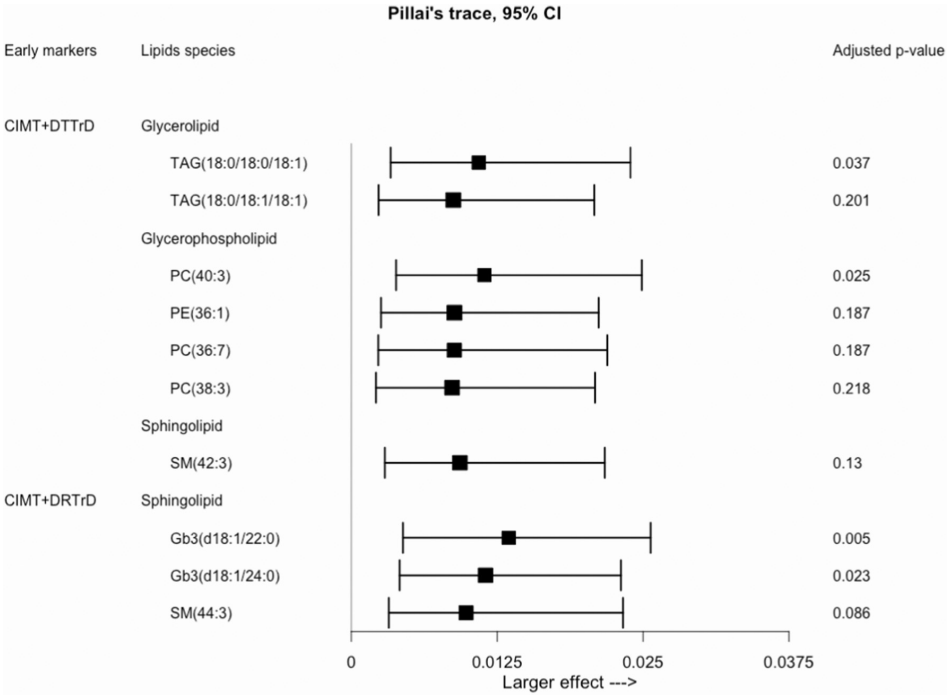

Fig. 2. Forest plot of the multivariate lipidome-wide association study of early markers of osteoporosis and atherosclerosis adjusted for age, sex, body mass index, smoking habit, physical activity and alcohol consumption. Analysis was performed for two different outcomes based on combination of early markers of atherosclerosis, carotid intima media thickness (CIMT); and osteoporosis, total mineral density of the distal tibia's trabecular bone (DTTrD) and distal radius's trabecular bone (DRTrD). The second column represents lipid species and the classes they belong to. Pillai's trace is the test statistic in multivariate analysis of variance (MANOVA) ranging from 0 to 1. Confidence interval of the test statistic was calculated from 100 bootstraps of the original data. *P*-values were adjusted using Bonferroni's method.

For early markers of osteoporosis, we focused mainly on the bone mineral density measured from metabolically active trabecular-rich distal site of weight-bearing tibia (DTTrD) and non-weight-bearing radius (DRTrD). Based on our results, mostly glycerolipids and glycerophospholipids are jointly associated with DTTrD and CIMT. On the other hand, mostly sphingolipids seem to be associated with DRTrD and CIMT. We speculate that these differences might be attributed to the differences in weight-bearing functionality between radius and tibia. The four glycerolipids associated with DTTrD and CIMT: *TAG(18:0/18:0/18:1)*, *TAG(18:0/18:1/18:1)*, *TAG(16:0/18:0/18:1)* and *TAG (16:0/16:0/18:1)* differ from each other in the structure of their fatty acyls side chains. Elevated triacylglyceride (TAG) level is independent risk factor for cardiovascular disease after controlling for low-density lipoprotein and high-density lipoprotein cholesterol [30]. For example, *TAG(54:2)*, triacylglyceride with fatty acyls of 54 carbons and 2 double bonds has been shown to be a strong predictor for cardiovascular disease [31]. Similarly, *TAG(56:6)*, triacylglyceride with fatty acyls of 56 carbons and 6 double bonds, has been shown to be associated with development of cardiovascular disease in type 2 diabetes mellitus [32]. TAG might promote atherosclerotic plaque through endothelial dysfunction, inflammation and thrombosis mechanisms via atherogenic remnant particles and apo C-III, a proinflammatory and proatherogenic protein [33]. Various studies have shown positive correlation between TAG and bone mineral density [34–37]. Dragojevič et al. reported that osteoporotic tissue cells exhibit higher osteoclastogenesis and lower TAG metabolism. This leads to speculation that perhaps TAG plays protective role in osteoporosis [38].

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are glycerophospholipids with choline and ethanolamine head respectively. PCs are major components of cell membrane and play role in cell signalling, food and energy storage and glycerophospholipid metabolism [39]. PEs are enriched in the inner leaflet of cell membrane and are involved in protein biogenesis, oxidative phosphorylation, membrane fusion, mitochondrial stability and autophagy [39]. Lysophosphatidylcholines, a metabolite of PCs, are reportedly significantly increased in the plasma of osteoporotic mice and is likely to increase reactive oxygen species which leads to oxidative stress damage [40,41]. This increase in oxidative stress may facilitate extensive bone loss and bone fragility and thereby exacerbating the process of osteoporosis [42]. Furthermore, oxidative stress may promote endothelial dysfunction, thereby promoting a vascular inflammatory response which leads to the progression of atherosclerosis [43]. Studies have shown that PCs with lower levels of carbon atoms might be associated with cardiovascular disease [44]. For example, *PC(34:1),* a phosphatidylcholine with 34 carbons and 1 double bonds is related to ischemia [45]. Also, PEs and PCs along with TAGs have been shown to be associated with osteoporosis [46].

Sphingomyelins, *SM (44:3)* and *SM (43:3)* and globotriasoylceramides, *Gb3(d18:1/24:0)* and *Gb3 (d18:1/22:0)* are sphingolipids that were significantly associated with the early markers of both osteoporosis and atherosclerosis. Sphingolipids are involved in important biological processes such as proliferation, migration and apoptosis [47]. Sphingosine 1-phosphate (S1P), an intermediate in sphingolipid metabolism, is a potent mediator of bone homeostasis and acts as a coupling factor between osteoclast and osteoblast activity [48,49]. S1P, however, possesses both protective and harmful effects in the pathogenesis of

atherosclerosis [50,51]. S1P maintains the endothelial integrity by promoting endothelial barrier function [50], whereas also induce inflammation and thrombosis in atherosclerosis [51]. Sphingomyelins, the most common sphingolipids in mammalian cells and tissues are independently associated with coronary artery disease [52]. Sphingomyelins are also crucial for mineralization process in healthy bones [53]. Fabry disease, an X-linked inborn error of glycosphingolipid catabolism, leads to accumulation of globotriaosylceramide in body fluids. Increase in globotriaosylceramide is associated with osteopenia and accelerated bone resorption of the lumbar spine and femoral neck [54]. Globotriaosylceramide also induces oxidative stress, inflammation [55] and endothelial dysfunction [56] in Fabry patients.

This study complements our previous system-level lipidomics based osteoporosis-atherosclerosis study [16] by identifying thirteen additional novel lipid species (*AcylCarnitine.13.0.*, *AcylCarnitinr.18.2.*, *PC (36:1)*, *PC(36:7)*, *PC(40:3)*, *PC(34.1)*, *PC(39.6)*, *PC(37.6)*, *PE(36:2)*, *Gb3(d18:1/22:0)*, *Gb3(d18:1/24:0)*, *SM(42:3),SM(44:3)*) jointly associated with early markers of both the diseases. The study also replicated the three most significant lipid species identified by the system-level approach (*TAG(18:0/18:0/18:1)*, *TAG(18:0/18:1/18:1)*, *TAG(16:0/18:0/18:1)*) confirming their potential joint role in osteoporosis-atherosclerosis comorbidity.

There were certain limitations in our study. The study was based on a relatively young population cohort with an early phase of cardiovascular disease and osteoporosis and very few clinically diagnosed cases. However, we believe that our results unveiled suggestive joint associations between the molecular lipid species and early markers of the diseases that warrants further research in a case–control setting that includes participants with clinically diagnosed osteoporosis and atherosclerosis. Sex-stratified associations between lipid species and early markers of osteoporosis and atherosclerosis remained undetected with statistical significance because of the lack of sufficient statistical power. Furthermore, all study participants are of European origin. Further research in a case–control setting with populations of different ethnicities are needed.

5. Conclusion

Our results from lipidome-wide multivariate association analysis of early markers of osteoporosis and atherosclerosis support the osteoporosis-atherosclerosis comorbidity hypothesis. Specifically, this study identified *TAG(18:0/18:0/18:1)*, *PC(40:3)*, *Gb3(d18:1/22:0) and Gb3(d18:1/24:0)* as the most significant molecular lipid species jointly associated with early markers of both the diseases. These new molecules may provide potential new biomarkers for the prediction of these comorbid conditions.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2021.116030.

Data availability statement

The dataset supporting the conclusions of this article were obtained from the Cardiovascular Risk in Young Finns Study which comprises health related participant data. The use of data is restricted under the regulations on professional secrecy (Act on the Openness of Government Activities, 612/1999) and on sensitive personal data (Personal Data Act, 523/1999, implementing the EU data protection directive 95/46/EC). Due to these restrictions, the data cannot be stored in public repositories or otherwise made publicly available. Data access may be permitted on a case-by-case basis upon request only. Data sharing outside the group is done in collaboration with YFS group and requires a data-sharing agreement. Investigators can submit an expression of interest to the chairman of the publication committee Professor Mika Kähönen (Tampere University, Finland) or Professor Terho Lehtimäki (Tampere University, Finland).

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Ethical approval

The study was approved by the ethical committee of the Hospital District of Southwest Finland on 20 June 2017 (ETMK:68/1801/2017), and all participants have given an informed written consent. Data protection will be handled according to current regulations.

CRediT authorship contribution statement

Binisha H. Mishra: Conceptualization, Investigation, Writing – original draft. **Pashupati P. Mishra:** Supervision, Data curation, Writing – review & editing. **Nina Mononen:** Writing – review & editing. **Mika Hilvo:** Writing – review & editing. **Harri Sievänen:** Writing – review & editing. **Markus Juonala:** Writing – review & editing. **Marika Laaksonen:** Writing – review & editing. **Nina Hutri-Kähönen:** Writing – review & editing. **Jorma Viikari:** Writing – review & editing. **Mika Kähönen:** Writing – review & editing. **Olli T. Raitakari:** Writing – review & editing. **Reijo Laaksonen:** Writing – review & editing. **Terho Lehtimäki:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

None.

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PUBLICATION IV

Gene set analysis of transcriptomics data identifies new biological processes associated with early markers of atherosclerosis but not with those of osteoporosis: atherosclerosis-osteoporosis co/multimorbidity study in the Young Finns Study

Mishra, Binisha H.; Sievänen,Harri; Raitoharju,Emma; Marttila,Saara; Mononen,Nina;; Viikari,Jorma; Juonala,Markus; Laaksonen,Marika; Hutri-Kähönen,Nina; Kähönen,Mika; Raitakari,Olli T.; Lehtimäki,Terho; Mishra, Pashupati P

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Gene set analysis of transcriptomics data identifies new biological processes associated with early markers of atherosclerosis but not with those of osteoporosis: Atherosclerosis-osteoporosis co/multimorbidity study in the Young Finns Study

Binisha H. Mishra [a,b,c,*], Harri Sievänen [d], Emma Raitoharju [e,f], Nina Mononen [a,b,c], Jorma Viikari [g,h], Markus Juonala [g,h], Marika Laaksonen [i], Nina Hutri-Kähönen [j], Mika Kähönen [b,k], Olli T. Raitakari [l,m,n], Terho Lehtimäki [a,b,c], Pashupati P. Mishra [a,b,c]

[a] Department of Clinical Chemistry, Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland
[b] Finnish Cardiovascular Research Center Tampere, Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland
[c] Department of Clinical Chemistry, Fimlab Laboratories, Tampere, Finland
[d] The UKK Institute for Health Promotion Research, Tampere, Finland
[e] Molecular Epidemiology, Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland
[f] Tampere University Hospital, Tampere, Finland
[g] Department of Medicine, University of Turku, Turku, Finland
[h] Division of Medicine, Turku University Hospital, Turku, Finland
[i] Fazer Lab Research, Oy Karl Fazer Ab, Helsinki, Finland
[j] Department of Paediatrics, Tampere University Hospital, Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland
[k] Department of Clinical Physiology, Tampere University Hospital, Tampere, Finland
[l] Research Centre of Applied and Preventive Cardiovascular Medicine, University of Turku, Turku, Finland
[m] Department of Clinical Physiology and Nuclear Medicine, Turku University Hospital, Turku, Finland
[n] Centre for Population Health Research, University of Turku and Turku University Hospital, Turku, Finland

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ABSTRACT

Aim: We aimed at identifying the shared biological processes underlying atherosclerosis-osteoporosis co/multimorbidity.

Methods: We performed gene set analysis (GSA) of whole-blood transcriptomic data to identify biological processes shared by the early markers of these two diseases. Early markers of diseases, carotid intima-media thickness (CIMT) for atherosclerosis and trabecular bone mineral density (BMD) from distal radius and tibia for osteoporosis, were used to categorize the study participants into cases and controls. Participants with high CIMT (>90th percentile) were defined as cases for subclinical atherosclerosis. Study population-based T-scores for BMD were calculated and T-score ≤ -1 was used for the definition of low BMD cases i.e., early indicator of osteoporosis.

Results: We did not identify any gene sets jointly associated with early markers of atherosclerosis and osteoporosis. We identified three novel and replicated 234 gene sets significantly associated with high CIMT with false discovery rate (FDR) ≤ 0.01. Only two genes, both related to the immune system, were identified to be associated with high CIMT by traditional differential gene expression analysis. However, none of the studied gene sets or individual genes were significantly associated with tibial or radial BMD. The three novel CIMT associated gene sets contained genes involved in copper homeostasis, neural crest cell migration and nicotinate and nicotinamide metabolism. The 234 replicated gene sets in this study are related to the immune system, hypoxia and apoptosis, consistent with the existing literature on atherosclerosis.

* Corresponding author. Faculty of Medicine and Health Technology, Tampere University, Department of Clinical Chemistry, Fimlab Laboratories, Finnish Cardiovascular Research Center, Tampere, 33520, Finland.
E-mail address: binisha.hamalmishra@tuni.fi (B.H. Mishra).

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Conclusions: This study identified novel biological processes associated with high CIMT but not with reduced BMD.

1. Introduction

Atherosclerosis and osteoporosis, both complex and multifactorial diseases, are growing public health challenges with a major impact on disease management and health care costs globally [1–3]. Osteoporosis, meaning excessively porotic bone, is a skeletal disease that causes weak and fracture-prone bones. Atherosclerosis is thickening or hardening of the arteries due to accumulation of plaque in the inner lining obstructing blood flow,a which may result in myocardial infarction or stroke. Both diseases often progress without symptoms until a major clinical event such as myocardial infarction due to atherosclerosis or bone fracture due to osteoporosis. Therefore, early detection of these diseases at subclinical phase is crucial for devising preventive measures and managing health care costs to prevent catastrophic health spending, especially in developing countries [4].

Several studies have suggested that osteoporosis and atherosclerosis are co/multimorbidities as they share common pathophysiological mechanisms, molecular pathways and risk factors such as inflammatory cytokines, lipid oxidation products, vitamin D and K deficiency, bone and vascular mineralization, estrogen deficiency and air pollution [5–9]. Further investigations involving omics data are crucial for understanding the shared mechanisms underlying these diseases at multiple biological levels. For instance, investigation of transcriptomics data in the context of broader biological themes such as biological processes or pathways using a gene set analysis (GSA) method may lead to the development of novel tools and research directions for improved and holistic prevention, diagnosis, and treatment of the diseases, as shown in other studies [10,11].

Most of the transcriptomics studies on osteoporosis or atherosclerosis are based on traditional differential gene expression (DGE) analysis followed by overrepresentation-based GSA of the identified list of differentially expressed genes for pre-determined gene sets representing biological processes and pathways [12–16]. Gene sets representing biological processes and pathways are usually derived from databases such as Kyoto Encyclopedia of Genes and Genomes [17], Gene Ontology [18] or integrative resources such as Molecular Signatures Database (MSigDB) [19]. A major limitation of the traditional overrepresentation-based GSA is that it requires an ambiguous threshold for determining differentially expressed genes and the results may vary with different threshold levels [20]. Development of advanced threshold-free GSA methods is an active field of research due to its usefulness in interpreting transcriptomics as well as other omics data [21–25]. These state-of-the-art GSA methods utilize the whole transcriptome data instead of a list of genes and are therefore statistically more powerful. The methods fall into two major categories based on the null hypothesis tested: self-contained or competitive [20]. A self-contained GSA method tests whether genes in a given gene set are more differently expressed than expected. A competitive GSA method tests whether genes in a given gene set are more differently expressed than the other genes in the analyzed data. Therefore, while a competitive GSA method compares the differential expression of the analyzed gene sets to all the genes except the genes in the analyzed set, a self-contained method tests differential expression of each of the analyzed gene sets independently. Self-contained GSA is particularly useful in an exploratory study such as this one due to high statistical power as compared to competitive GSA [20].

This study is a continuation of our previous studies on atherosclerosis-osteoporosis co/multimorbidity where we identified clustering-based lipidomic and transcriptomic modules as well as individual molecular lipid biomarkers jointly associated with early markers of both the diseases [26–28]. The main objective of the present study was to identify transcriptome-wide gene sets representing biological processes that are jointly associated with early markers of both osteoporosis and atherosclerosis. To achieve the stated goal, we performed self-contained GSA of the transcriptomic data, in addition to the traditional DGE analysis, concerning bone mineral density (BMD) as an early trait of osteoporosis and carotid intima-media thickness (CIMT) as an early trait of atherosclerosis.

2. Materials and methods

2.1. Study population

This study was based on the Young Finns Study (YFS), one of the largest existing longitudinal studies into cardiovascular health from childhood to adulthood with regular follow-ups from 1980 onwards [29]. The study was initiated in 1980 with 3,596 children and adolescents aged 3–18 years. The participants were randomly selected from the areas of five university hospitals in Finland (Turku, Tampere, Helsinki, Kuopio, and Oulu) and have been followed up for over 40 years. Out of 2200 participants from the 27-year follow-up in 2007, we included those for whom the measurements of early markers of both osteoporosis and atherosclerosis as well as transcriptomic data were available. The current study is, thus, based on 1,093 participants, aged 31–45 years, from the 2007 follow-up (Table 1), with one atherosclerotic and two osteoporotic markers as summarized in Table 2. The genome-wide transcriptome of the study participants was profiled from whole-blood samples collected from the 2011 follow-up. The YFS was approved by the 1st Ethical committee of the Hospital District of Southwest Finland and by local ethical committees (1st Ethical Committee of the Hospital District of Southwest Finland, Regional Ethics Committee of the Expert Responsibility area of Tampere University Hospital, Helsinki University Hospital Ethical Committee of Medicine, The Research Ethics Committee of the Northern Savo Hospital District and Ethics Committee of the Northern Ostrobothnia Hospital District) on 20 June 2017 (ETMK:68/1801/2017). The study protocol of each study phase corresponded to the proposal by the World Health Organization. All participants gave written informed consent and the study was conducted in accordance with the Helsinki declaration. At prior YFS follow-ups, informed consent of every participant under the age of 18 years was obtained from a parent and/or legal guardian. Data protection will be handled according to current regulations.

2.2. Measurement of early markers of osteoporosis

Trabecular BMD from the metabolically active trabecular-rich distal (5%) site of weight-bearing tibia (DTTrD in mg/cm^3) and distal (4%) site of non-weight-bearing radius (DRTrD) were determined with peripheral quantitative computed tomography (pQCT) according to standard procedures [30] and used as an early marker of osteoporosis, as described elsewhere [26]. The same pQCT device was used in all five centres (XCT 2000R, Stratec, Medizintechnik GmbH, Pforzheim, Germany). Precision of pQCT measurements in this multicentre study was evaluated by performing repeated scans of volunteers in each centre before starting and after completing the measurements. Radius and tibia were measured among 39 women and men twice with repositioning. Reproducibility (coefficient of variation, CV%) was 0.5% for DTTrD and 1.6% for DRTrD [30].

Table 1
Characteristics of the study population concerning subclinical atherosclerosis (high CIMT).

	Cases	Controls	*p*
Number of subjects (%)	107 (10%)	986 (90%)	–
Sex (female %)	34%	58%	–
Age, years	41 (±4)	38 (±5)	<0.0001
Body mass index, kg/m^2	28(±5)	26 (±5)	<0.0001
Total cholesterol (mmol/l)	5.2 (±1)	5.0 (±0.9)	0.15
LDL cholesterol (mmol/l)	3.2 (±0.9)	3.1 (±0.8)	0.11
HDL cholesterol (mmol/l)	1.2 ±(0.3)	1.4 ±(0.3)	<0.0001
Triglycerides (mmol/l)	1.7 ±(1)	1.3 (±0.9)	0.001
Serum glucose (mmol/l)	5.6 (±0.9)	5.3 (±0.9)	0.003
Insulin (IU/l)	12.9 (±14.9)	8.5 (±7.4)	0.003
C-reactive protein (mg/l)	2.5 (±9.6)	1.8 (±3.3)	0.43
Systolic blood pressure (mmHg)	129 (±15)	119 (±14)	<0.0001
Diastolic blood pressure (mmHg)	81 (±12)	75 (±11)	<0.0001
Participants with hypertension (%)	17/106 (16%)	46/984 (5%)	<0.0001
Alcohol consumption, units/day	1.1 (±1.5)	0.9 (±1.5)	0.25
Physical activity index (MET-h/wk)	18 (±19)	20 (±22)	0.38
Daily smoking, %	15/107 (14%)	136/986 (14%)	1
Daily calcium intake (mg)	1314 (±606)	1256 (±526)	0.34
Daily vitamin D intake (μg)	8.5 (±4.7)	7.8 (±4)	0.18
Family risk factor for coronary heart disease (%)	26/107 (24%)	154/986 (16%)	0.03
Participants with osteoporosis (%)	2/106 (2%)	7/983 (1%)	0.49
Participants with bone fractures (%)	43/107 (40%)	368/986 (37%)	0.63
Participants with family history for osteoporosis (%)	6/107 (6%)	51/977 (5%)	1
Usage of corticosteroids at least once a month (%)	7/107 (7%)	49/986 (5%)	0.64
Carotid intima-media thickness (CIMT) (average, mm)	0.84(±0.08)	0.61(±0.07)	–
Participants with CIMT > 1 mm	5/107 (5%)	0/986	–
Distal radius trabecular bone mineral density (DRTrD) (mg/cm^3)	234 (±36)	225 (±36)	0.009
Distal tibia trabecular bone mineral density (DTTrD) (mg/cm^3)	251 (±34)	240 (±34)	0.003

Participants with high CIMT (>90th percentile) were defined as cases and the rest as controls. Data are mean (± standard deviation) or proportions (%) and statistical significance (*p*) of the difference between the cases and controls.

Table 2
Characteristics of the study population concerning subclinical osteoporosis (low BMD).

	Cases	Controls	*p*
Number of subjects (%)	176 (16%)	917 (84%)	–
Sex (female %)	60%	45%	–
Age, years	39 (±5)	38 (±5)	0.01
Body mass index, kg/m^2	24(±4)	26 (±5)	<0.0001
Total cholesterol (mmol/l)	5.1 (±0.9)	5.0 (±0.9)	0.12
LDL cholesterol (mmol/l)	3.1 (±0.8)	3.1 (±0.8)	0.7
HDL cholesterol (mmol/l)	1.4 ±(0.3)	1.3 ±(0.3)	<0.0001
Triglycerides (mmol/l)	1.3 ±(0.7)	1.4 (±0.9)	0.05
Serum glucose (mmol/l)	5.3 (±1)	5.3 (±0.9)	0.69
Insulin (IU/l)	7.1 (±6.2)	9.3 (±8.9)	0.0001
C-reactive protein (mg/l)	1.4 (±2.4)	1.9 (±4.6)	0.04
Systolic blood pressure (mmHg)	119 (±15)	120 (±14)	0.32
Diastolic blood pressure (mmHg)	74 (±12)	76 (±11)	0.09
Participants with hypertension (%)	7/175 (4%)	56/915 (6%)	0.35
Alcohol consumption, units/day	1.0 (±1.4)	0.9 (±1.5)	0.3
Physical activity index (MET-h/wk)	16 (±18)	20 (±22)	0.002
Daily smoking, %	35/176 (20%)	116/917 (13%)	0.02
Daily calcium intake (mg)	1181 (±489)	1277 (±542)	0.02
Daily vitamin D intake (μg)	7.8 (±4.2)	7.9 (±4)	0.75
Family risk factor for coronary heart disease (%)	35/176 (20%)	145/917 (16%)	0.22
Participants with osteoporosis (%)	3/175 (2%)	6/914 (1%)	0.34
Participants with bone fractures (%)	70/176 (40%)	341/917 (37%)	0.25
Participants with family history for osteoporosis (%)	10/174 (6%)	47/910 (5%)	0.91
Usage of corticosteroids at least once a month (%)	11/176 (6%)	45/917 (5%)	0.58
Carotid intima-media thickness (CIMT) (average, mm)	0.62(±0.1)	0.63(±0.1)	0.46
Participants with CIMT > 1 mm	2/176 (1%)	3/917 (0.5%)	0.4
Distal radius trabecular bone mineral density (DRTrD) (mg/cm^3)	195 (±27)	232 (±35)	<0.0001
Distal tibia trabecular bone mineral density (DTTrD) (mg/cm^3)	195 (±15)	250 (±30)	<0.0001

The YFS population-based T-scores for trabecular BMD from distal tibia were calculated and T-score ≤ -1 was used to define cases (low BMD). Data are mean (± Standard Deviation) or proportions (%) and statistical significance (*p*) of the difference between the cases and controls. (For characteristics of the study population concerning subclinical osteoporosis based on T-scores for trabecular BMD from distal radius, see Supplementary Table S2.)

2.3. Measurement of early markers of atherosclerosis

Carotid intima-media thickness (CIMT) was used as an early marker of atherosclerosis, as described elsewhere [26]. An ultrasound imaging device with a high-resolution system (Sequoia 512, Acuson) including 13.0 MHz linear array transducers was used for CIMT measurement by trained sonographers following a standardized protocol. The image was focused on the posterior (far) wall, and images were recorded from the angle showing the greatest distance between the lumen–intima interface and the media–adventitia interface. A scan including the beginning of the carotid bifurcation and the common carotid artery was recorded and stored in digital format on optical discs for subsequent off-line analysis. All scans were analyzed by one reader blinded to the participants' details. The best-quality end-diastolic frame was selected. Several measurements of the common carotid far wall were taken approximately 10 mm proximally and mean CIMT was used as the outcome. For reproducibility of the CIMT measurements, we re-examined 60 participants 3 months after the initial visit (2.5% random sample). The between-visit CV% of CIMT measurements was 6.4%. For reproducibility of the CIMT image analysis, 113 scans were re-analyzed by a second observer; CV% was 5.2%.

2.4. Health and lifestyle data

Physical activity index, based on weekly metabolic equivalent hours (MET-h/wk), was calculated from information on the frequency, intensity and duration of physical activity including leisure-time physical activity and commuting to the workplace. One MET corresponds to the energy consumption of one kilocalorie per kilogram of weight per hour at rest [31]. Participants' alcohol consumption information was assessed from self-reported on their alcohol consumption during the previous week where one unit is equivalent to 14 g of alcohol [32].

2.5. Blood transcriptomic analysis

Whole-genome transcriptome was profiled from whole-blood samples collected from the YFS participants during the 2011 follow-up. Expression levels were analyzed with Illumina HumanHT-12 version 4 Expression BeadChip (Illumina Inc.), containing 47,231 expression and 770 control probes. Samples with fewer than 6,000 significantly detected expression probes (detection $p < 0.01$) were discarded. Raw Illumina summary probe-level data was exported from Beadstudio and processed in R (http://www.r-project.org/) using a nonparametric background correction, followed by quantile normalization with control and expression probes, with the neqc function in the *limma* package [33] and a log2 transformation. Nine samples were excluded due to sex mismatch between the recorded sex and predicted sex based on RPS4Y1-2 and XIST mRNA levels on the Y and X chromosomes, respectively. After quality control, expression data were available for 1,654 samples, including 4 technical replicates, which were used to examine batch effects and subsequently excluded before further analysis.

2.6. Genotyping and genotype imputation

Genomic DNA was extracted from peripheral blood leukocytes using a commercially available kit and Qiagen BioRobot M48 Workstation according to the manufacturer's instructions (Qiagen, Hilden, Germany). Genotyping was done using custom build Illumina Human 670 k BeadChip atWelcome Trust Sanger Institute. Genotypes were called using Illuminus clustering algorithm. Samples that failed Sanger genotyping pipeline quality control criteria (i.e., duplicated samples, heterozygosity, low call rate, or Sequenom fingerprint discrepancy) were excluded from the analysis. Similarly, samples with sex discrepancy, low genotyping call rate (<0.95) and possible relatedness (pi-hat>0.2) were excluded from the analysis. Short Nucleotide Polymorphisms (SNPs) were excluded based on Hardy-Weinberg equilibrium test (p ≤ 1e-06), failed missingness test (call rate<0.95) and failed frequency test (minor allele frequency<0.01). Overall, 546677 genotyped SNPs passed the quality control. Genotype imputation was performed using Minimac3 [34] and 1000G phase3 reference set on Michigan Imputation Server.

2.7. Biostatistical analysis

Skewness in the values for body mass index (BMI), physical activity and alcohol consumption was corrected with log2 transformation. CIMT value higher than or equal to 90th percentile was used for the definition of high CIMT as an early indicator of atherosclerosis. Next, we calculated the YFS population-based T-scores for trabecular BMD at distal radius and tibia. The T-score represents the magnitude of deviation of a participant's BMD from BMD of an average healthy 31–45 years old people of the same sex. Similar to our earlier studies, T-score ≤ -1, was used for the definition of cases for low BMD as an early indicator of osteoporosis [30]. The definition of cases for low BMD was based on both distal tibia and distal radius using the corresponding reference values [30] and analysis was repeated for both distal tibia and distal radius-based case-control setup. DGE analysis and GSA was performed on the residuals left after performing a regression analysis of transcriptomic data against age, sex, BMI, physical activity index (MET), smoking habit, alcohol consumption and blood cell counts of erythrocytes, leukocytes and thrombocytes. DGE analysis concerning early markers of both osteoporosis and atherosclerosis separately was performed using moderated *t*-test implemented in Linear Models for Microarray Data (*limma*) R/Bioconductor package [35]. GSA was performed using rotation gene set test (*ROAST*) [22] implemented in *limma* R/Bioconductor package. Latest version of curated gene sets (c2.all.v7.4) were downloaded from MSigDB [19]. *ROAST* is a self-contained gene set test that tests whether any of the genes in the set are differentially expressed [20]. Instead of permutation of sample labels, *ROAST* uses rotation, a Monte Carlo technique for multivariate regression, for *p*-value (*p*) estimation [36]. Sex-stratified GSA was performed to identify sex-specific associations between the studied gene sets and the studied traits. Potential modification of effect of the identified biological processes on the early markers of atherosclerosis and osteoporosis by sex was tested by analyzing regression models of the studied early markers (in both categorized and continuous forms) against eigengene of the analyzed gene set (summary expression level of a gene set calculated as the first principal component of the member genes), eigengene and sex interaction, sex, age, BMI, smoking habit and alcohol consumption habit. DGE and GSA were performed using the R environment for statistical computing, version 3.6.0 [37].

Associations between identified gene sets and the studied early markers were validated with Mendelian randomization (MR) approach using weighted genetic risk scores (wGRS) for the gene sets as their genetic instruments. The wGRSs were calculated using PLINK v.190b3 software [38] from genetic data of the studied participants [Section 2.6] using the independent SNPs associated with each contributing gene to the gene sets with FDR <0.05 selected from a recent expression quantitative trait loci (eQTL) study of blood gene expression [39]. The genetic risk scores were weighted with the effect sizes of the selected SNPs on the corresponding genes obtained from the study by Ref. [39]. Highly correlated SNPs were excluded from the calculation of wGRS by performing pruning with a window size of 200 genetic variants, sliding across the genome with step size of 50 variants at a time, and filter out any SNPs with linkage disequilibrium (LD) r2>0.25. Ambiguous SNPs were removed. Mismatching SNPs were resolved by strand-flipping the alleles to their complementary alleles. We first assessed the strength of the wGRSs as genetic instruments for the gene sets by testing wGRS—gene set associations. Eigengenes of the gene sets were used as summary expression levels for the association analyses. We then performed MR analyses using the wGRSs as instrumental variables, eigengenes as exposures and the early markers as outcomes, through instrumental variable regressions using *ivreg* R package [40]. Similar

approach has been described also elsewhere [41]. The instrumental variable regressions were performed with both continuous and category forms of the studied early markers as outcomes. All the instrumental variable regression analyses were adjusted for age, sex, BMI, MET, smoking and alcohol consumption habit.

3. Results

3.1. Study population characteristics

Population characteristics and summary statistics of the early markers of atherosclerosis and osteoporosis of the studied population are shown in Tables 1 and 2 and Supplementary Table S2 respectively. Number of diseases are based on self-reports [30]. Among the traditional risk factors listed in the tables, both high CIMT and low BMD were significantly associated with age, body mass index, HDL-cholesterol, triglycerides, and insulin.

3.2. Association between early markers of atherosclerosis and osteoporosis

The early marker of subclinical atherosclerosis (CIMT) had a weak but significant ($p < 0.01$) positive correlation (r) with the early markers of osteoporosis ($r = 0.13$ with DRTrD and $r = 0.1$ with DTTrD).

3.3. Differential gene expression analysis related to reduced BMD and high CIMT

Two genes, *RDH8* (retinol dehydrogenase 8) and *CFAP74* (cilia and flagella associated protein 74) both with FDR of 0.04 were identified to be associated with high CIMT using traditional gene-wise differential expression analysis. No genes were identified to be associated with low BMD with FDR<0.05.

3.4. Gene set analysis related to reduced BMD and high CIMT

Among the 6290 studied gene sets, we identified three novel gene sets (Fig. 1) and replicated 234 gene sets (Fig. 2, and Supplementary Table S1) significantly associated with high CIMT with FDR ≤0.01. The member genes of the three novel gene sets as well as of all but three replicated gene sets had decreased average expression level among individuals with high CIMT as compared to controls (Fig. 1). For comparison, with a more liberal threshold of FDR <0.05, we obtained 1799 gene sets associated with high CIMT. However, no gene sets were identified to be associated with reduced trabecular BMD with FDR <0.05 with either of the distal tibia and distal radius-based case-control analyses. Similarly, no gene sets were identified to be associated with the studied traits in the sex-stratified analyses. Effect modification due to sex on early markers of the diseases was not detected in the studied population, perhaps due to relatively young age of the studied population (Supplementary Table S3).

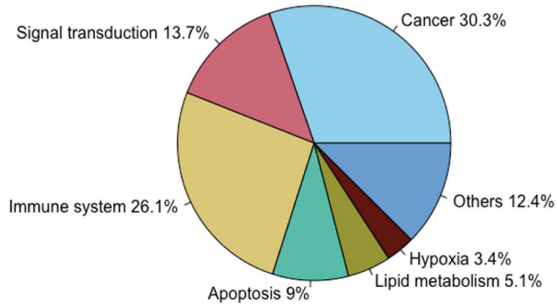

Fig. 2. Pie chart summarizing biological processes represented by the 234 gene sets replicated in this study to be significantly associated with high CIMT with FDR ≤0.01.
The category 'Others' includes gene sets involved in pentose phosphate pathway, proteoglycan biosynthesis, beta alanine metabolism, copy number variation, galactose catabolism, vitamin c ascorbate metabolism, pantothenate and CoA biosynthesis, necrosis and cellular processes.

3.5. Type 1 error test

We obtained large number of significant gene sets associated with high CIMT with GSA despite finding only two genes with traditional DGE analysis. While higher statistical power of GSA as compared to the traditional DGE analysis is expected, we tested whether the proportion of type 1 error or false positive results is unexpectedly high in our results. We did that by running *ROAST* on the null transcriptomics data

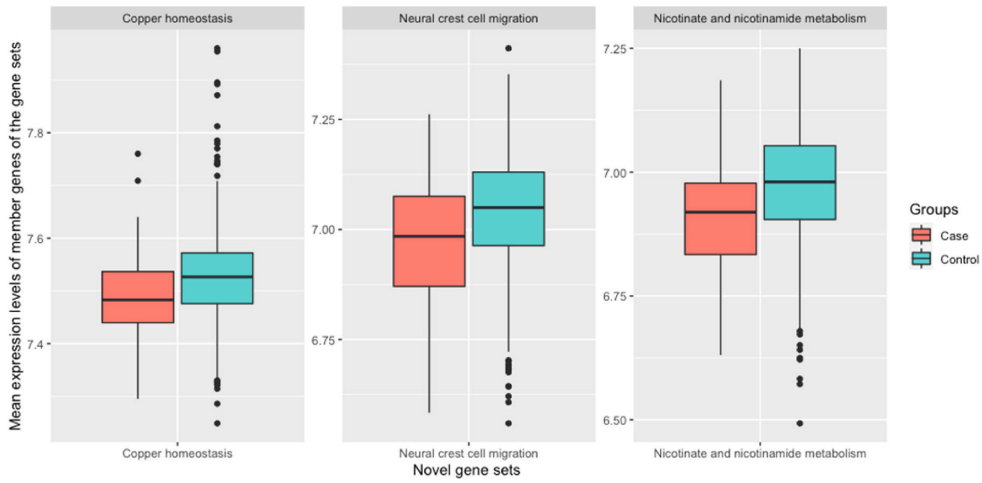

Fig. 1. Boxplots illustrating difference in mean expression levels of member genes of the three novel gene sets associated with high carotid intima media thickness (cases) as compared with controls.

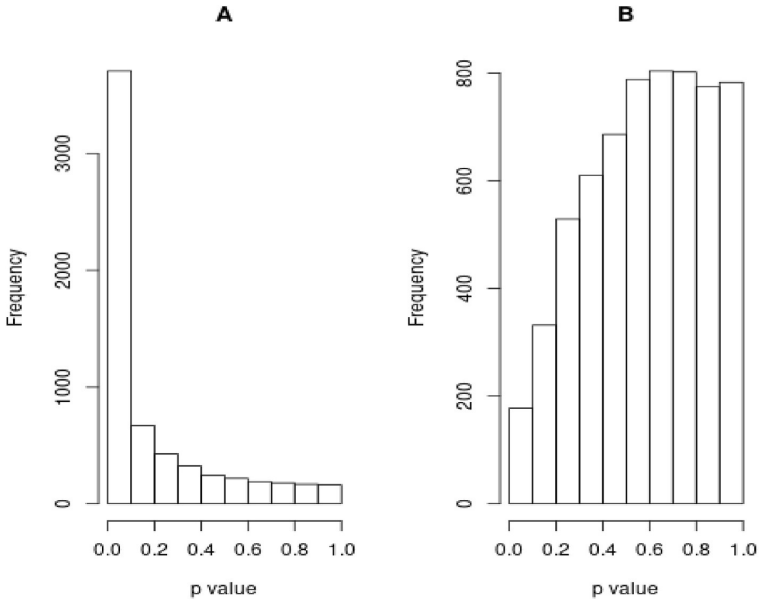

Fig. 3. Histogram of *p* obtained from the used gene set analysis (GSA) method, *ROAST*, with original gene expression data (A) and randomized (null) gene expression data with no true differential gene expression (B).
The plot indicates that the chosen method, *ROAST,* is conservative for false positive results.

generated by randomizing the sample labels of the original analyzed transcriptomics data. The distribution of estimated *p* of gene sets from the null data was then compared to the one from original data. Distribution of *p* from null data is expected to have uniform distribution. Our results showed that *ROAST* generated a slightly left skewed distribution of *p* from null transcriptomics data (Fig. 3) which suggests that the method is conservative in generation of false positive results.

3.6. Mendelian randomization analysis

We identified statistically significant wGRS—gene set associations ($p < 0.05$) for the gene sets representing copper metabolism ($p = 0.03$) and nicotinate and nicotinamide metabolism ($p = 0.003$), but not for neural crest cell migration ($p = 0.73$). However, we did not find evidence of causal effect of the gene set expression levels on the early markers of the diseases. For detailed results from MR analyses using instrumental variable regression, see Supplementary Table S4.

4. Discussion

In this study, we performed GSA of whole-blood transcriptomic data from the YFS participants to identify biological processes associated with early markers of osteoporosis (pQCT-based DTTrD and DRTrD) and atherosclerosis (ultrasound based CIMT). We implemented self-contained GSA that tests whether a set of biologically related genes is differentially expressed between compared groups irrespective of the other genes in the genome. The most used GSA methods in similar research are either overrepresentation-based methods such as GOstat [42] or competitive methods such as GSEA [21] that tests whether a set of biologically related genes is differentially expressed between compared groups relative to all the other genes in the genome. Self-contained GSA is suitable in an exploratory study such as this one that aims to identify all the biological processes associated with a disease rather than the most interesting biological processes among the relevant ones. With self-contained GSA of transcriptomics data from the YFS participants using curated gene sets from MSigDB, we identified three novel gene sets and replicated 234 gene sets significantly associated with high CIMT with FDR $\leq$0.01. Identification of a large number of significant gene sets associated with high CIMT with GSA despite finding only two genes with traditional DGE analysis highlights the importance of self-contained GSA in exploratory research. However, no gene sets or genes were identified to be significantly associated with low trabecular BMD. These results also indicate that the biological processes represented by the identified 237 gene sets are altered already in the early phase of atherosclerosis but not so with osteoporosis.

One of the three novel gene sets identified to be associated with high CIMT is related to copper homeostasis which is known to play an important role in cardiovascular diseases. Our results indicate that genes involved in copper homeostasis are on average downregulated among people with high CIMT which may, in turn, affect the copper concentration in blood. Several biochemical studies have shown an association between altered serum copper concentration and cardiovascular disease [43,44]. Alterations in copper homeostasis can lead to dyslipidemia [45] which plays role in both osteoporosis [46] and atherosclerosis [47]. For example, elevated serum copper has been shown to be associated with increased serum concentrations of total cholesterol and HDL cholesterol [48]. Copper is a prooxidative metal that stimulates oxidative modifications of LDL-cholesterol and participates in the oxidation of LDL within the arterial walls. Oxidized LDL is taken up by macrophages which is then transformed into foam cell in the artery wall, which is the hallmark of atherosclerosis.

Another novel gene set, among the three, contained genes involved in nicotinate and nicotinamide metabolism. Nicotinamide is an antioxidant that plays a key role in the production of nicotinamide adenine dinucleotide responsible for maintaining redox homeostasis and modulating the immune response [49]. Antioxidants maintain redox haemostasis by eliminating reactive oxygen species (ROS) [50]. ROS molecules trigger oxidative stress thereby promoting endothelial dysfunction via vascular inflammatory response leading to progression of atherosclerosis [51]. Oxidative stress can also cause extensive bone

loss and bone fragility and thereby exacerbating the process of osteoporosis [52].

The third novel gene set identified in this study contains genes involved in neural crest cell (NCC) migration. NCC is a multipotent cell population that migrates to generate diverse differentiated cell types such as coronary artery smooth muscles cells, skeletal and connective tissue components depending on their origin, where they migrate and settle. For example, cardiac neural crest originates from postotic hindbrain and plays role in the formation of the outflow tract endocardial cushions [53]. Preotic NCCs are capable of osteogenic and chondrogenic differentiation and therefore might be related to the pathogenesis and progression of coronary artery diseases and bone disease [54,55] Alterations in activities of genes involved in NCCs migration among participants with high CIMT, as identified in this study, support the hypothesis that NCCs may play role in atherosclerosis.

We performed Mendelian randomization analysis for validation of the observed associations between the three novel gene sets and the studied outcomes (CIMT, BMD) using wGRS as genetic instrumental variables for the gene sets. No evidence for causal effect of the gene sets' expression level on the studied early markers of the diseases was found. However, we speculate that the MR analyses in this study is under powered due to small sample size of the studied cohort and use of early markers of the diseases instead of the clinical outcomes. This study raises novel hypotheses and warrants further studies for confirmation.

The replicated 234 gene sets identified to be associated with high CIMT in this study are related to the immune system, hypoxia and apoptosis, consistent with the existing literature. Immune and inflammatory response plays an important role in the pathogenesis of both atherosclerosis and osteoporosis [56]. Hypoxia plays a key role in the progression of atherosclerotic plaque by promoting lipid accumulation, foam cell formation, inflammation and angiogenesis [57]. Several studies have demonstrated that hypoxia promotes osteoclast differentiation and activity [58,59]. Similarly, apoptosis plays a crucial role in the pathogenesis of atherosclerosis as well as osteoporosis [60,61].

There were certain limitations to the study. The study was based on a relatively young population cohort and therefore limited to the subclinical phase of atherosclerosis and osteoporosis with only few clinically diagnosed cases of cardiovascular disease and osteoporosis. While the bone and carotid artery measurements were taken from the 2007 follow-up, the analyzed transcriptomic data was profiled from the whole blood samples collected from the 2011 follow-up. Therefore, the study was based on valid assumptions that there was no substantial change in bone and carotid artery traits [62–64] over a four-year period among a healthy adult population. The study was based on microarray technology because for a large epidemiological study such as the one, it was the cost-efficient choice at the time of follow-up (the year 2011). We also acknowledge that usage of whole blood gene expression data has its limitations in fully capturing bone specific biological processes. However, blood gene expression profile might recapitulate biological processes in bone marrow because immune cells within blood migrate back and forth between blood and bone marrow and are known to influence bone homeostasis [65]. Therefore, the approach can provide biomarkers that are easily assessable and non-invasive as compared to bone tissue. Furthermore, as all the study participants are of European origin, studies with populations of different ethnicities are needed.

4.1. Conclusion

This study identified three novel gene sets and replicated 234 known gene sets significantly associated with high-CIMT. The gene sets represent three different biological processes– copper homeostasis, neural crest cell migration and nicotinate and nicotinamide metabolism– which might explain the transcriptomic link between the biological processes and atherosclerosis and serve as its biomarkers. Additionally, the study highlights the importance of using self-contained GSA for exploratory transcriptomics studies.

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CRediT author contribution statement

Binisha H. Mishra: Conceptualization, investigation, data analysis, writing - original draft; Harris Sievänen: Data acquisition, reviewed and edited the manuscript; Emma Raitoharju: reviewed and edited the manuscript; Nina Mononen: reviewed and edited the manuscript; Jorma Viikari: Data acquisition, reviewed and edited the manuscript; Markus Juonala: reviewed and edited the manuscript; Marika Laaksonen: Data acquisition; Nina Hutri-Kähönen: Data acquisition; Mika Kähönen:Data acquisition, reviewed and edited the manuscript; Olli T. Raitakari: Data acquisition, reviewed and edited the manuscript; Terho Lehtimäki: Data acquisition, reviewed and edited the manuscript; Pashupati P. Mishra: Conceptualization, supervision, data analysis, reviewed and edited the manuscript.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2022.10.005.

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