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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

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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.

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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 ($p \leq 1e\text{-}06$), 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).

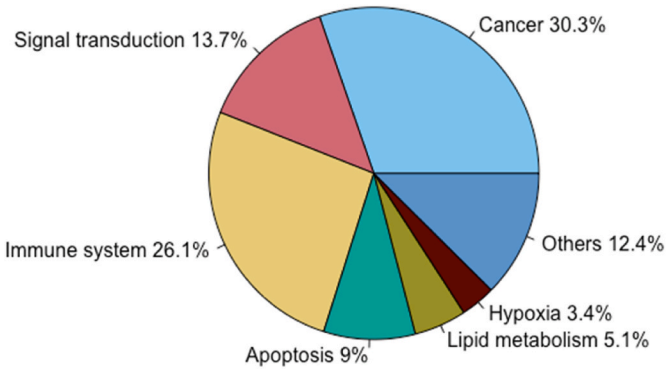

Fig. 2. Pie chart summarizing biological processes represented by the 234 gene sets replicated in this study to be significantly associated with high CIMT with FDR ≤0.01.
The category 'Others' includes gene sets involved in pentose phosphate pathway, proteoglycan biosynthesis, beta alanine metabolism, 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

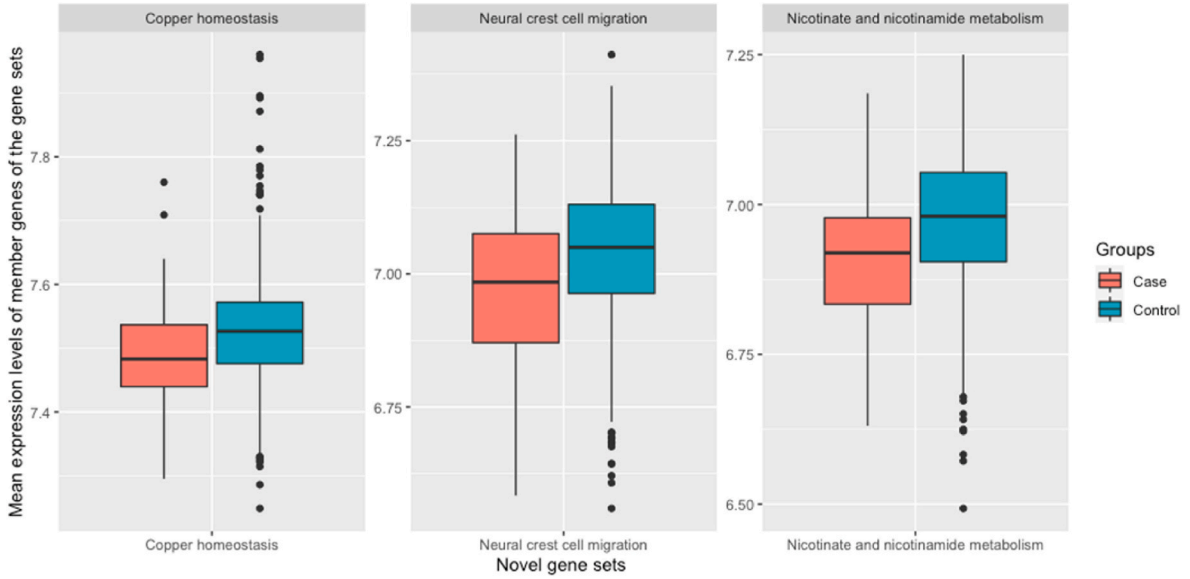

Fig. 1. Boxplots illustrating difference in mean expression levels of member genes of the three novel gene sets associated with high carotid intima media thickness (cases) as compared with controls.

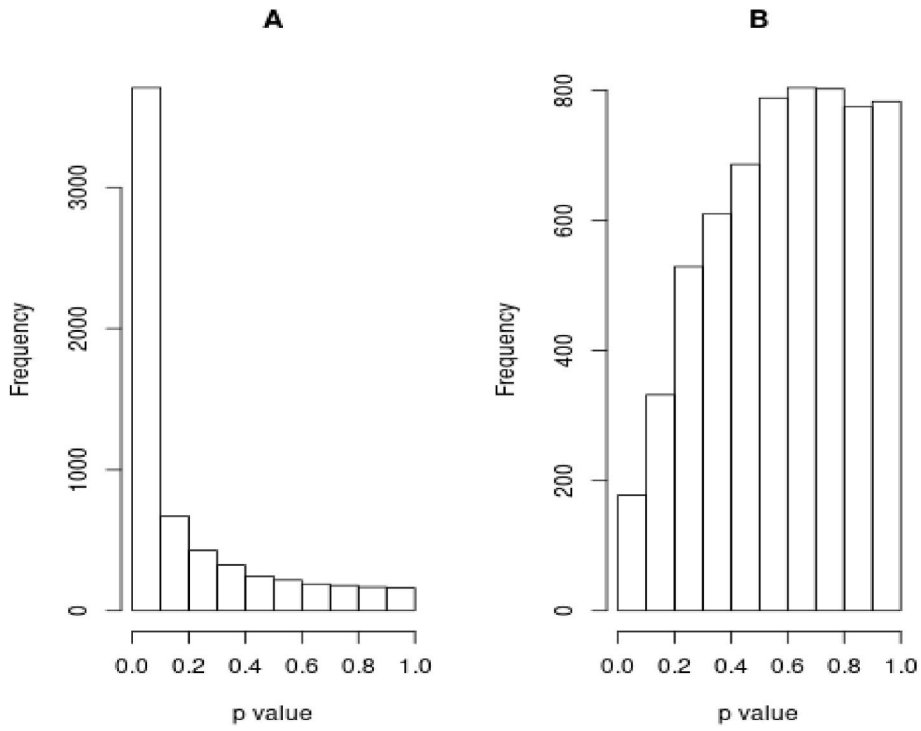

Fig. 3. Histogram of *p* obtained from the used gene set analysis (GSA) method, *ROAST*, with original gene expression data (A) and randomized (null) gene expression data with no true differential gene expression (B).
The plot indicates that the chosen method, *ROAST,* is conservative for false positive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