Original Research *Featured Article*

Transcriptional landscape analysis identifies differently expressed genes involved in follicle-stimulating hormone induced postmenopausal osteoporosis

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Abstract

Osteoporosis is a disorder associated with bone tissue reorganization, bone mass, and mineral density. Osteoporosis can severely affect postmenopausal women, causing bone fragility and osteoporotic fractures. The aim of the current study was to compare blood mRNA profiles of postmenopausal women with and without osteoporosis, with the aim of finding different gene expressions and thus targets for future osteoporosis biomarker studies. Our study consisted of transcriptome analysis of whole blood serum from 12 elderly female osteoporotic patients and 12 non-osteoporotic elderly female controls. The transcriptome analysis was performed with RNA sequencing technology. For data analysis, the edgeR package of R Bioconductor was used. Two hundred and fourteen genes were expressed differently in osteoporotic compared with non-osteoporotic patients. Statistical analysis revealed 20 differently expressed genes with a false discovery rate of less than 1.47×10^{-4} among osteoporotic patients. The expression of 10 genes were up-regulated and 10 down-regulated. Further statistical analysis identified a potential osteoporosis mRNA biomarker pattern consisting of six genes: *CACNA1G*, *ALG13*, *SBK1*, *GGT7*, *MBNL3*, and *RIOK3.* Functional ingenuity pathway analysis identified the strongest candidate genes with regard to potential involvement in a follicle-stimulating hormone activated network of increased osteoclast activity and hypogonadal bone loss. The differentially expressed genes identified in this study may contribute to future research of postmenopausal osteoporosis blood biomarkers.

Keywords: Bone, transcriptome, age, musculoskeletal, female, biomarkers

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Introduction

Osteoporosis (OP) is a skeletal fragility disorder characterized by low bone mineral density (BMD), modification of bone tissue microarchitecture quality, and susceptibility to sudden fractures.[1] Osteoporotic fractures, including those of the hip and spine, are often causes of a poor quality of life, disability, and increased risk of mortality among patients.[2] Every year the prevalence of OP increases globally, resulting in new healthcare and financial concerns.[3]

Postmenopausal women face the largest challenge of bone loss, due to changes in levels of reproductive hormones.[4,5] Postmenopausal osteoporosis (PMOP) is associated with a decrease of estrogen (ESR) and an increase of follicle-stimulating hormone (FSH) and luteinizing hormone (LH).[6] FSH influences bone mass both indirectly and directly, via ESR and an extracellular signal-regulated kinase–mitogen-activated protein kinase (Erk/Mek) signaling pathway with Gi2a stimulation of MEK/Erk, the nuclear factor κβ (NF-κβ) and 3-kinase-Akt, respectively.[7]

An understanding of OP mechanisms is crucial for effective disease prevention, diagnosis, and therapy. OP diagnosis and fracture risk estimation is based on a BMD scale (T-score <-2.5 SD); however, OP fractures might occur among those at a moderate risk.[8–10]

Evaluation of bone quality and fracture risk remains a research area of great interest. Previous studies have concentrated on miRNA signatures and bone turnover biochemical markers of OP.[11–13] Whole genome RNA sequencing (RNA-seq) is a powerful tool for investigating the pathological pathways of complex disorders. To the best of our knowledge, there are no previous studies of whole blood mRNA transcriptome analysis among

Table 1 Patients and controls.

(a) Characteristics of healthy control group individuals (KR) and OP patients (OP)

ID	Age	Weight	Height	BMI	Total hip (T-score)	Total hip (Z-score)	L1-L4 (T-score)	L1-L4 (Z-score)	ID	Age	Weight	Height	BMI	Total hip (T-score)	Total hip (Z-score)	L1-L4 (T)	L1-L4 (Z)
KR1	71	62	169.7	21.53	−0.7	0.8	−0.7	1.1	OP1	71	70,3	160	27.46	−1.2	−0.6	−2.9	−1.6
KR2	61	97.9	164.7	36.09	1.2	1.4	0.1	0.3	OP2	62	63.2	159	25.00	−2.6	−1.6	−2.6	1.2
KR3	62	70	156.2	28.69	−0.7	0.2	−0.7	0.5	OP3	63	57	157	23.12	−2.5	−1.3	−3.8	−2.2
KR4	65	72	154.5	30.16	1.2	2.2	−0.3	1	OP4	65	55	166.8	19.77	−2.5	−1.2	−3.9	−2.1
KR5	75	75	162.4	28.44	0.1	1.4	0.2	1.7	OP5	75	83	163	31.24	−2.5	−1.2	−2.2	−1
KR6	76	78	168.6	27,44	−0.7	0.6	1	2.4	OP6	77	82	166	29.76	−0.3	0.8	−2.6	−1.1
KR7	76	63	162	24,01	−0.3	1.4	0.5	2.4	OP7	77	79	163	29.73	−0.4	0.5	−2.5	−1.4
KR8	73	76	163.3	28,50	−0.9	0.3	0.8	2.1	OP8	73	72	160	28.13	−2.7	−1.6	−3.8	−2.4
KR9	65	79	161.6	30,25	0.7	1.5	0.2	1.3	OP9	66	71	165	26.08	−3	−2	−2.2	−0.9
KR10	80	51	152	22,07	−0.9	1.3	0.8	3.1	OP10	80	65	149	29.28	−1.4	0	−3.7	−2
KR11	71	66.2	156.5	27.03	−0.7	0.7	−0.2	1.4	OP11	71	50	158	20.03	−1.2	0.2	−2.9	−1.1
KR12	67	74	167	26.53	−0.6	0.4	1.4	2.8	OP12	67	54.7	154	23.06	−2.5	−1	−2.3	−0.4
KR mean	**70.2**	**72.01**	**161.54**	**27.59**	**−0.19**	**1.02**	**0.26**	**1.66**	OP mean	**70.6**	**66.85**	**160.07**	**26.09**	**−1.9**	**−0.75**	**−2.95**	**−1.25**

(b) Mean values for subgroups of OP patients: group A (OP only in lumbar spine), B (lumbar spine and total hip OP)

ID	Age	Weight	Height	BMI	Total hip (T-score)	Spine, L1-L4 (T-score)
Group A	75.2	69.26	159.2	27.25	−0.9	−2.92
Group B	67.3	65.13	160.7	25.20	−2.61	−2.97

BMI: body mass index.
Mean values are shown in bold.

postmenopausal osteoporotic patients. Total blood mRNA shares about 80% of transcriptome with other major tissues.[14] mRNA reflects the functional state of cells, and integrates responses to both genetic and epigenetic factors of gene regulation, making it a promising way to explore disease progression.[15–17]

In the present study, we report our whole blood RNA-seq transcriptome analysis of 12 elderly postmenopausal osteoporotic and 12 elderly non-osteoporotic females. Differential expression analysis was combined with functional network annotation. As a result, we found a pattern of differently expressed genes (DEGs) that are potentially involved in direct FSH osteoclastogenesis and a bone resorption activation pathway. Our findings might be of interest as new targets for future research of PMOP biomarkers that could result in more effective diagnosis and follow-up of OP.

Methods

Patients and controls

The selection of female individuals for transcriptome analysis was based on BMD. OP patients were selected from the bone densitometry database of the Clinic of Traumatology

Table 2 Candidate genes for osteoporosis mRNA biomarkers

Gene symbol	Gene name	Gene function	Gene location	FDR	*p*	logFC
CACNA1G	Calcium channel, voltage-dependent, T type, alpha 1G subunit	Calcium ion transport	17q22	7.75×10^{-69}	3.35×10^{-73}	2.502
ALG13	ALG13, UDP-N-acetylglucosaminyltransferase subunit	Catalyzes N-glycosylation, glycosyltransferase and deubiquitinase activities	Xq23	1.02×10^{-51}	8.86×10^{-51}	1.739
SBK1	SH3-Binding domain kinase 1	Serine-threonine protein kinase, involved into signal-transduction pathways	16p11.2	1.11×10^{-21}	1.44×10^{-25}	1.609
GGT7	Gamma-glutamyltransferase 7	Transpeptidation of aminoacids and metabolism of glutathione	20q11.22	1.11×10^{-15}	1.92×10^{-19}	1.702
RIOK3	RIO kinase 3	Serine-threonine protein kinase, involved into signal-transduction pathways	18q11.2	6.65×10^{-6}	1.44×10^{-9}	−8.330
C10orf71	Chromosome 10 open reading frame 71	Unknown	10q11.23	1.07×10^{-5}	2.78×10^{-9}	1.613
KDM6B	Lysine (K)-specific demethylase 6B	Regulates gene expression through histone demethylation	17p13.1	2.72×10^{-5}	8.24×10^{-9}	0.612
MBNL3	Muscleblind-like splicing regulator 3	Regulates pre-mRNA splicing	Xq26.2	3.63×10^{-5}	1.26×10^{-8}	−0.780
GOLGA8B	Golgin A8 family, member B	Unknown	15q14	3.72×10^{-5}	1.45×10^{-8}	1.241
TCP11L2	t-Complex 11, testis-specific-like 2	Unknown	12q23.3	5.75×10^{-5}	2.49×10^{-8}	−0.817
MBNL1	Muscleblind-like splicing regulator 1	regulates pre-mRNA splicing	3q25	8.18×10^{-5}	3.89×10^{-8}	−0.300
CCNI	Cyclin I	Regulates activity of CDK kinases	4q21.1	$8.47 \times 10^{-}5$	4.4×10^{-8}	−0.518
RPS11	Ribosomal protein S11	Components of ribosomal 40S subunit	19q13.3	9.89×10^{-5}	5.57×10^{-8}	−0.471
UBA52	Ubiquitin A-52 residue ribosomal protein fusion product 1	Structural unit of ribosome	19p13.1-p12	1.02×10^{-4}	6.16×10^{-8}	−0.764
FAM117A	Family with sequence similarity 117, member A	Unknown	17q21.33	1.03×10^{-4}	6.68×10^{-8}	−0.769
NXF1	Nuclear RNA export factor 1	mRNA nucleocytoplasmic transporter activity	11q12-q13	1.11×10^{-4}	7.68×10^{-8}	0.575
PRR9	Proline rich 9	Unknown	1q21.3	1.19×10^{-4}	9.13×10^{-8}	1.528
PPP1CB	Protein phosphatase 1, catalytic subunit, beta isozyme	Serine-threonine protein phosphatase involved into signal-transduction pathways	2p23	1.19×10^{-4}	9.26×10^{-7}	−0.462
RPS16	Ribosomal protein S16	Component of ribosomal 40S subunit	19q13.1	1.47×10^{-4}	1.45×10^{-7}	−0.654
NCAN	Neurocan	Calcium ion binding	19p12	1.47×10^{-4}	1.46×10^{-7}	1.742

Note: The false discovery rate (FDR), log fold change (logFC) and *p*-values for the candidate genes are listed.

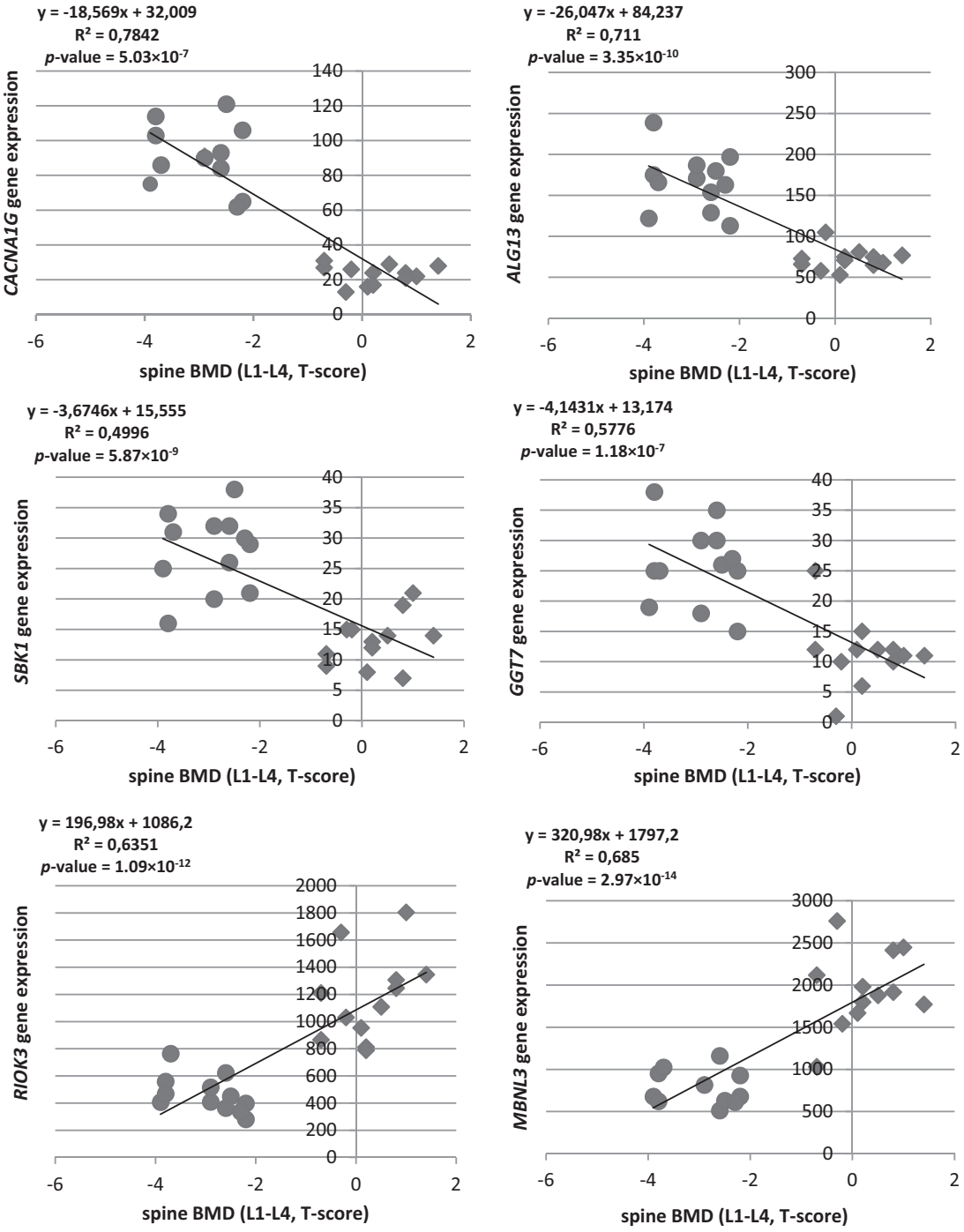

Figure 1 Correlation of candidate OP biomarkers gene expressions with spine BMD (T-score). Circles indicate OP patients; diamonds indicate controls. BMD: bone mineral density

and Orthopedics, Tartu University Hospital. All selected osteoporotic patients had relatively similar spine BMD T-scores (Table 1b).

We recruited a control group of 12 postmenopausal females with normal BMD from individuals who underwent densitometry testing during regular healthcare screening. Exclusion criteria for participation in the control group were a history of previous fractures and disease or medications that can affect bone quality. We performed age and BMI matching among patients and controls in order to reduce an influence of these factors on the transcriptome analysis. Mean age, height, weight, and body mass index (BMI) values were calculated for both the control (KO) and OP groups (Table 1a).

As half of the patients had low BMD only in spine and others in both spine and hip measured regions, we created two subgroups for further testing. Group A consisted of patients with OP only in the spine (T-score spine = −2.92 SD, hip 0.9 SD), and Group B with both lower spine (−2.97 SD) and hip (−2.61 SD) OP (Table 1b).

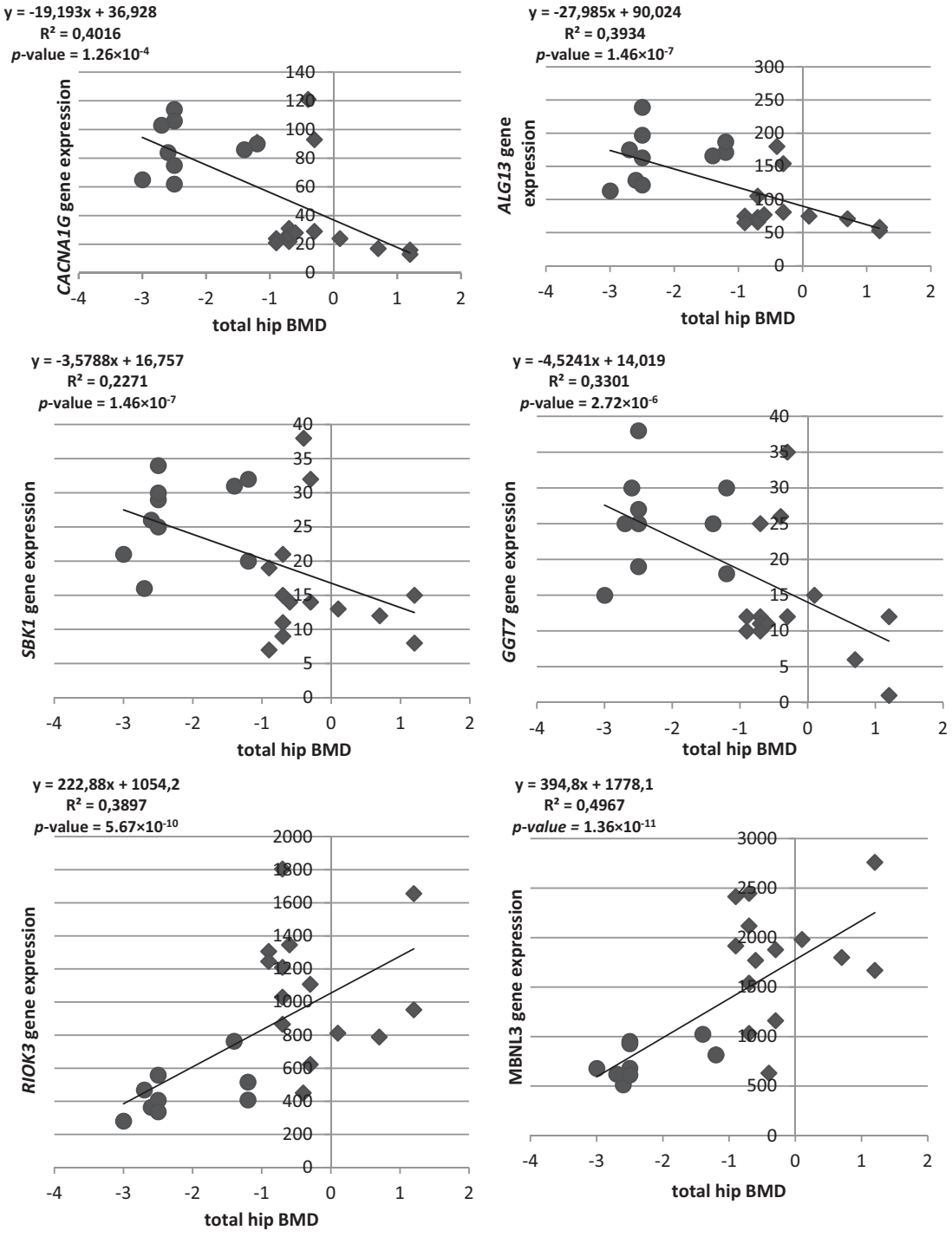

Figure 2 Correlation of candidate OP biomarkers gene expressions with total hip BMD (T-score). Circles indicate OP patients; diamonds indicate controls. BMD: bone mineral density

The protocols and informed consent form used in this study were approved by the Ethical Review Committee on Human Research of the University of Tartu (permit no. 221/M-34). All participants gave written informed consent.

Sample collection and RNA extraction

Tempus Blood RNA tubes (Applied Biosystems, Life Technologies Corp., Carlsbad, CA, USA) were used for collecting the samples of whole blood. Total RNA extraction from whole blood was achieved using a Tempus Spin RNA Isolation Kit (Ambion, Life Technologies Corp., Carlsbad, CA, USA). As total RNA from whole blood consists of up to 70% immunoglobulin mRNA, a GLOBINclear™ Kit (Ambion, Life Technologies Corp., Carlsbad, CA, USA) was applied to purify the samples of globin mRNA. The quality of total RNA was evaluated with an Agilent 2100 Bioanalyzer and RNA 6000 Nano kit (Agilent Technologies Inc., Santa Clara, CA, USA). The average RNA integrity number of the samples was at least 7.

Table 3 False discovery rate (FDR), log fold change (logFC) and *p*-values of mRNA biomarkers present in both A and B patient subgroups compared with healthy controls

	Group A vs. control group			Group B vs. control group		
Gene symbol	**FDR**	***p***	**logFC**	**FDR**	***p***	**logFC**
CACNA1G	3.86×10^{-42}	1.59×10^{-46}	2.489	1.70×10^{-49}	6.98×10^{-54}	2.508
ALG13	8.57×10^{-24}	7.03×10^{-28}	1.686	8.15×10^{-32}	6.69×10^{-36}	1.776
SBK1	1.15×10^{-10}	1.89×10^{-14}	1.651	2.08×10^{-11}	5.11×10^{-15}	1.585
GGT7	3.43×10^{-9}	7.03×10^{-13}	1.642	4.24×10^{-13}	6.96×10^{-17}	1.748
RIOK3	4.93×10^{-3}	5.87×10^{-6}	−0.692	8.17×10^{-9}	3.96×10^{-12}	−0.948
MBNL3	1.30×10^{-3}	1.12×10^{-6}	−0.738	3.95×10^{-7}	3.24×10^{-10}	−0.847

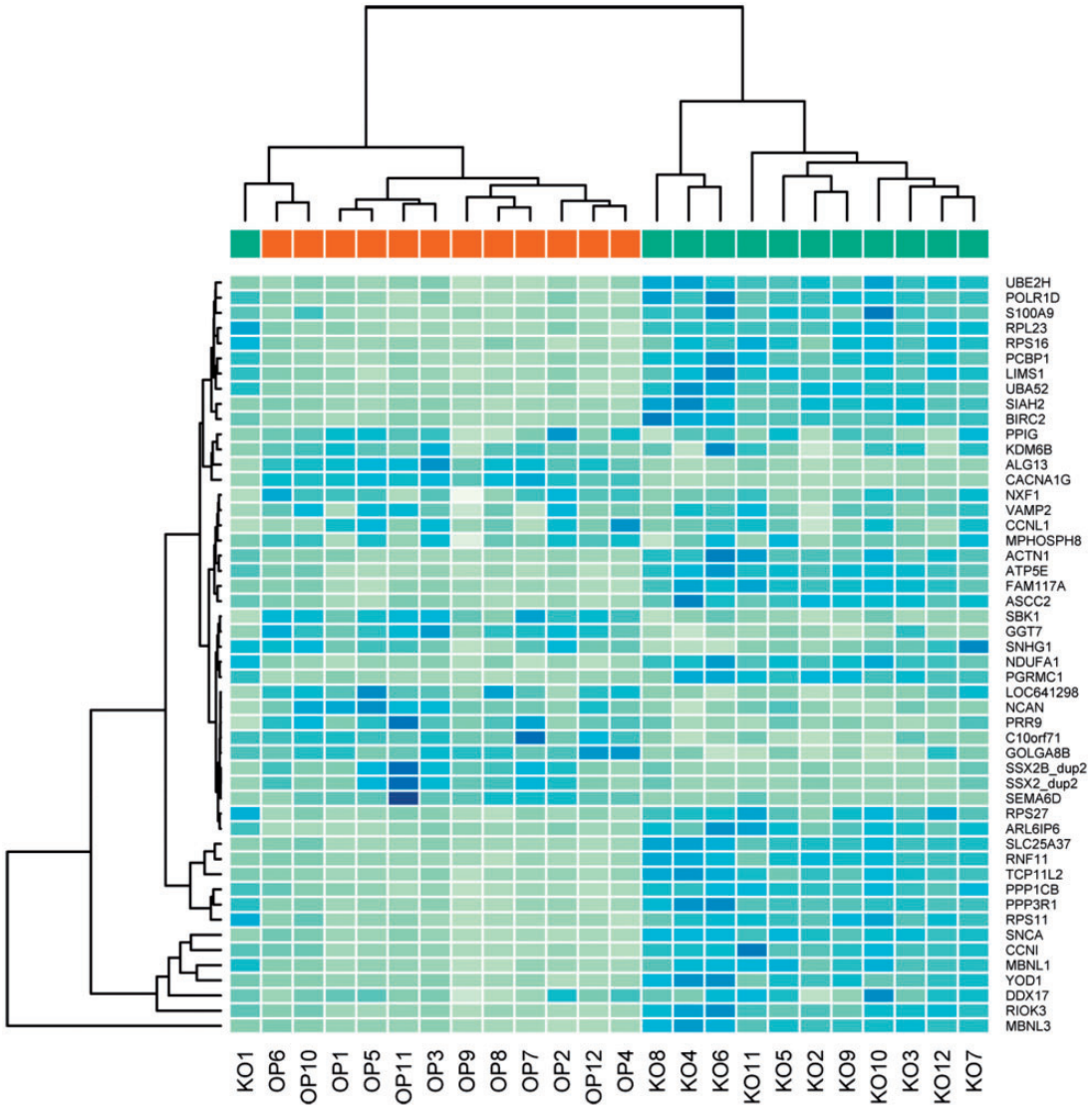

Figure 3 Heatmap analysis of differently expressed genes. The clustering according to gene expression between control samples (KO) and osteoporotic samples (OP) is observed. (A color version of this figure is available in the online journal)

Whole transcriptome RNAseq library preparation and sequencing

Fifty nanograms of each total RNA sample were amplified by applying the Ovation RNA-Seq System V2 (NuGen, Emeryville, CA, USA), after which SOLiD 5500 Wildfire (W) System chemistry (Life Technologies Corp., Carlsbad, CA, USA) was used to prepare the resulting cDNA for the DNA fragment library. Next, the 12 libraries were pooled together in equal amounts to construct two different library pools. The pooled libraries were converted to SOLiD 5500W

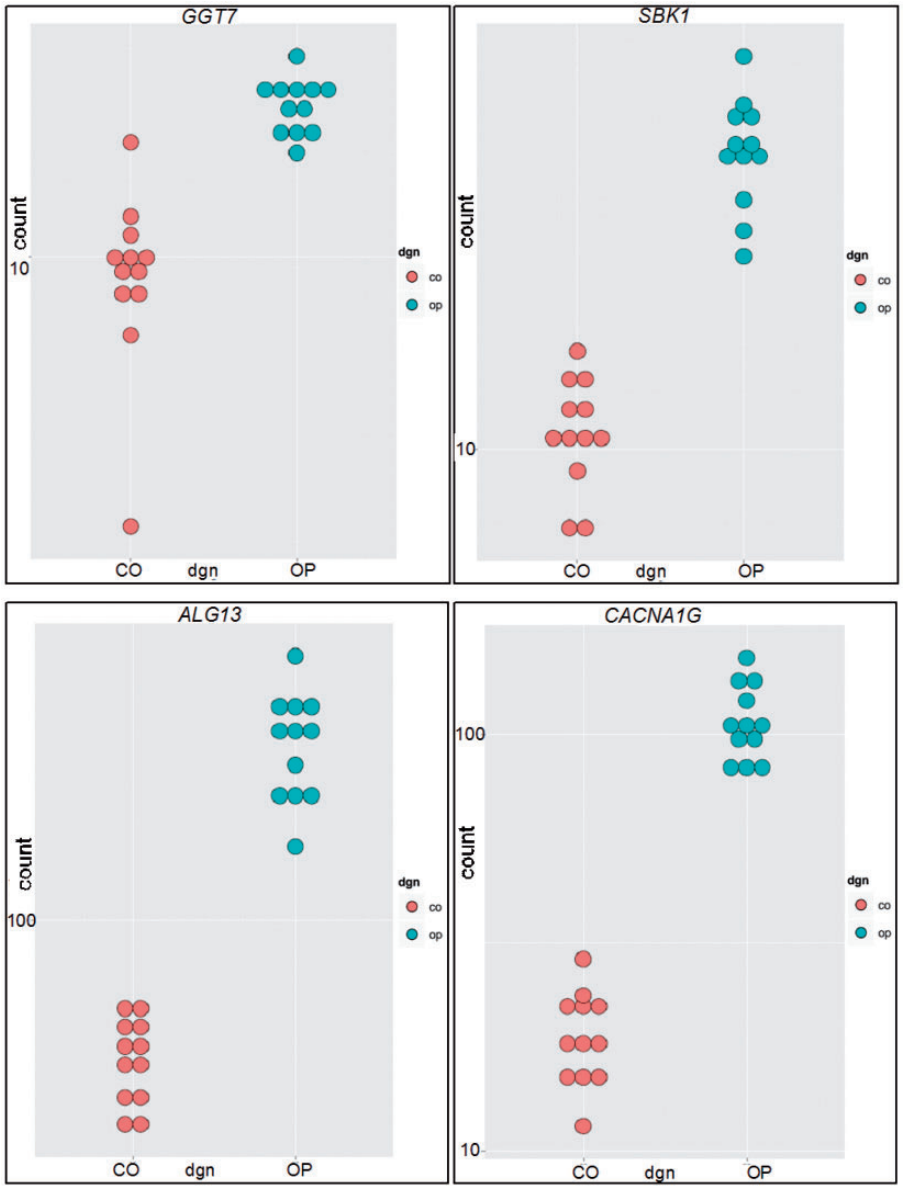

Figure 4 Differences in expressions of the strongest candidate OP biomarker genes between osteoporotic patients (turquoise) and healthy controls (red). (A color version of this figure is available in the online journal)

libraries, and sequencing was performed using a SOLiD 5500W platform and DNA sequencing chemistry (Life Technologies Corp., Carlsbad, CA, USA). Three-lane sequencing was applied and 12 libraries per lane were sequenced A total of 75 base pairs from a forward direction were sequenced, which altogether gave at least 30 million mappable reads per sample, i.e. sufficient for evaluation of the expression pattern of the transcriptome.

Statistical and functional analysis

Raw reads and whole transcriptome analysis workflow were mapped using Lifescope 2.5.1 software (Life Technologies Corp., Carlsbad, CA, USA). This workflow generates a very complex output, including gene and exon counts, alternative splicing, and fusion transcripts. For further analysis, we focused only on gene counts, because our primary question was related to the abundance of gene-targeted transcripts. For differential expression analysis, the R Bioconductor package edgeR was used, which implements exact statistical methods and generalized linear models for multigroup and multifactorial experiments.[18] A feature of edgeR is an empirical Bayes method that permits the estimation of gene-specific biological variation, even for experiments with minimal levels of biological replication. EdgeR can be applied to differential expression at gene, exon, or tag level. In our study, we used model-based normalization and applied a negative binomial model. Testing for differential expression was done using the exact test. Power analysis was performed with the RNAseqPS web tool.[19] Heatmap clustering analysis was generated with the gplots package in R. Network and pathway analysis was generated with QIAGEN's Ingenuity® Pathway

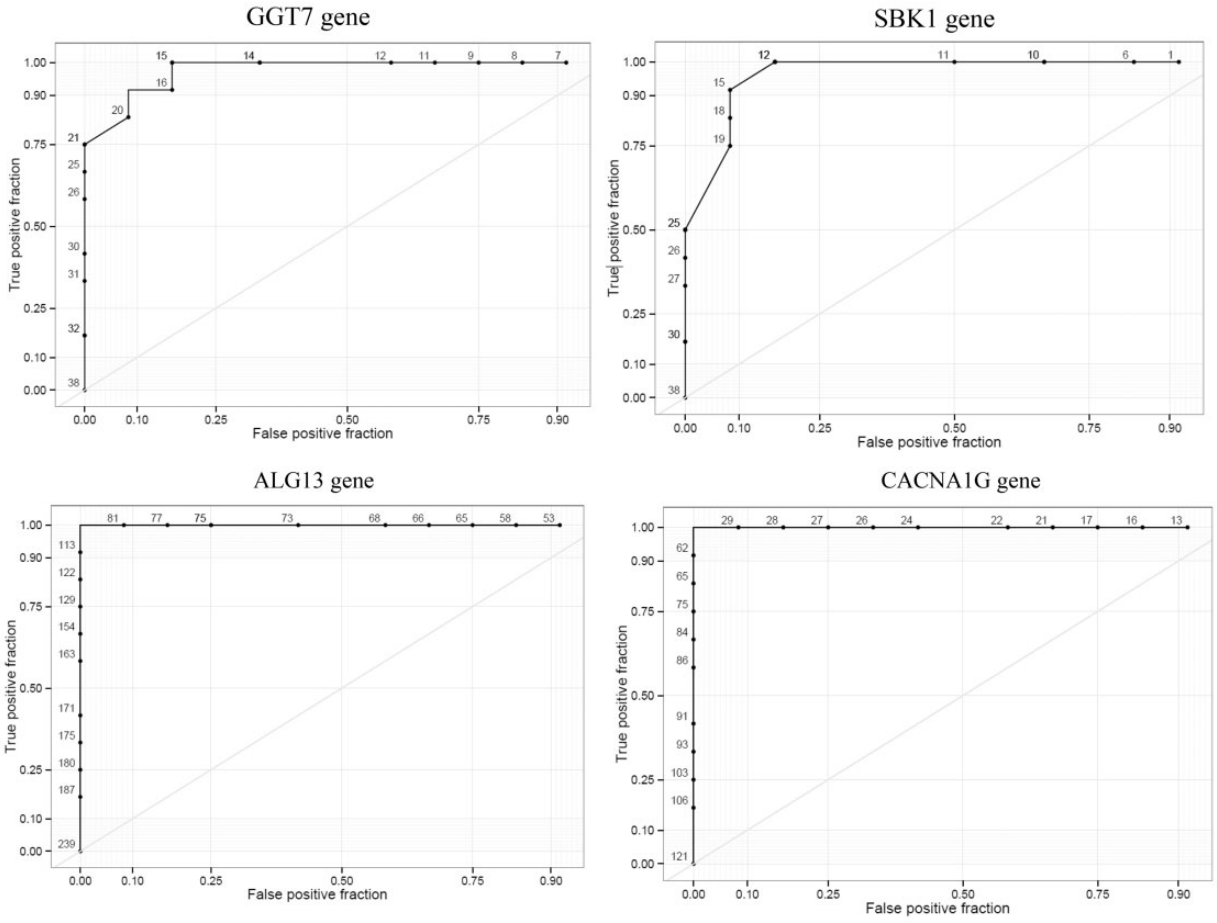

Figure 5 Mean ROC curves for the strongest candidate OP biomarkers gene expressions

Analysis (IPA®, QIAGEN Redwood City, CA, USA) software and the iPathwayGuide online tool (Advaita Plymouth, MI, USA).

Additionally, we tested the sensitivity of the DEGs as biomarker candidates of patients with OP, by comparing the mRNA patterns among OP patients' subgroups and controls using edgeR statistical analysis.

Results

Differential expression analysis

We performed whole blood mRNA-seq analysis to investigate the transcriptional profiles of non-OP and PMOP females. Whole transcriptome mRNA sequencing analysis identified 214 DEGs with a confidence level of a false discovery rate (FDR) of less than 0.05. Of the 214 genes, 154 were down-regulated. The 20 strongest DEGs (FDR less than 1.47×10^{-4}) present in the osteoporotic patients and absent from the control group are shown in Table 2. The expression of 10 DEGs was up-regulated and 10 down-regulated.

The statistical analysis of mRNA expression levels of the all OP patients using the edgeR Bioconductor package, revealed six candidate genes (*CACNA1G*, *ALG13*, *SBK1*, *GGT7*, *MBNL3*, and *RIOK3*) as the strongest candidates for a potential mRNA biomarker pattern indicative of OP. Subgroup analysis showed no significant difference in expression of the previously identified 20 candidate OP biomarkers between the subgroups.

Further analysis revealed a significant correlation between the expression of possible mRNA biomarkers and BMD values for OP patients compared with controls (Figures 1 and 2). The highest correlation of spine BMD was observed with the *CACNA1G* gene expression ($R^2 = 0.7842$, $p = 5.03 \times 10^{-7}$). Correlation between lumbar spine BMD and gene expression was more significant than the correlation between hip BMD and gene expression. On scatter plots of spine BMD, patient and control groups were clearly distinguishable (Figure 1). On scatter plots of hip BMD, three groups formed (controls, only spine OP, and spine and hip OP). Compared with the controls, the expression of genes among group B was more different than among group A. The FDR values of the potential mRNA OP biomarkers of subgroup B were also significantly lower than for group A (Table 3).

DEGs with statistically significant values were clustered with Heatmap analysis (Figure 3). The horizontal axis shows clustering within the two BMD groups (KO with high BMD, OP with low BMD). Differences between gene expression in the control (KO) and OP patient groups were clearly observed (Figures 3 and 4). The statistical power of the performed transcriptome analysis was represented using receiver operating characteristic curves for the four

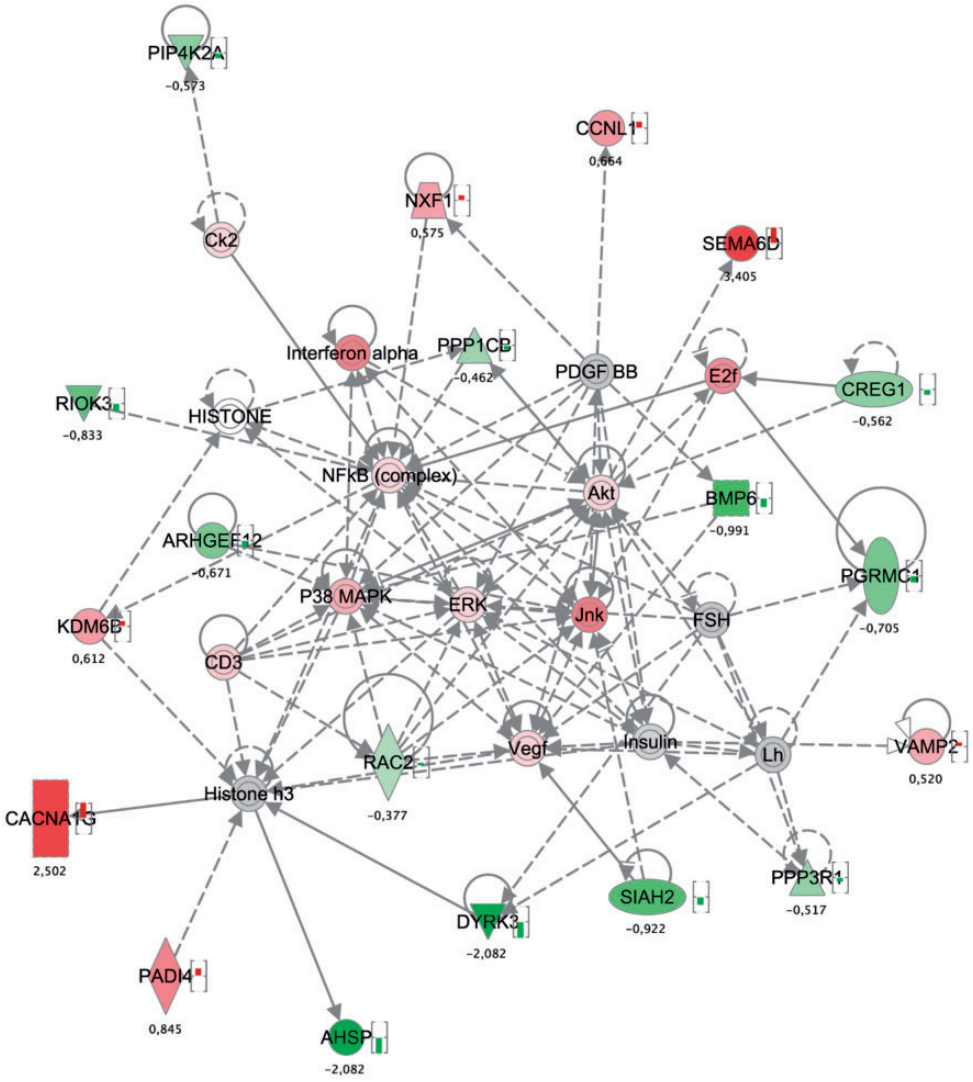

Figure 6 Genetic networks revealed among potential OP mRNA biomarkers with Ingenuity pathway Analysis software. The functional analysis identified involvement of the differently expressed genes into connective tissue disorders and the ''RNA Post-Transcriptional Modification, Molecular Transport, RNA Trafficking'' network. Down-regulated and up-regulated genes are highlighted in green and red colors, respectively. (A color version of this figure is available in the online journal)

strongest candidate genes (*GGT7, SBK1*, *ALG13*, and *CACNA1G*) (Figure 5). All four DEGs had high predictive power of an OP, with few or no false-positives. Accuracy for the strongest candidates *ALG13* and *CACNA1G* was 1.

Functional analysis

Network pathway analysis of the 20 genes with the highest FDR values revealed the potential involvement of the DEGs in the calcium signaling pathway and ERK/MAPK signaling pathways.

Involvement of the identified PMOP DEGs in these connective tissue disorder pathways was also found *in silico* using QIAGEN's Ingenuity software. IPA analysis showed that the DEGs were involved in cell growth and proliferation pathways, and molecular transport. IPA analysis also highlighted the involvement of the DEGs in the calcium signaling pathway and ERK/MAPK signaling pathway, Akt pathway, NF-κβ and FSH network (Figure 6).

Discussion

OP alters bone tissue metabolism pathways, which in our study manifested in changes in the mRNA levels of related genes in blood cells, and resulted in a special PMOP gene pattern of differential expression. Six candidate genes (*CACNA1G*, *ALG13*, *SBK1*, *GGT7*, *MBNL3*, and *RIOK3*) were the strongest candidates for a potential mRNA biomarker pattern indicative of OP in postmenopausal females. Subgroup analysis showed no significant difference of candidate gene expression between the subgroups, which is expected according to the basic knowledge of bone metabolism. OP affects entire skeleton, and areal BMD differences are connected to differences of bone shape, cortical-trabecular frame and metabolic activity, influenced by physical activity and lifestyle of the individual.

The strongest candidate gene was the alpha 1G subunit of the voltage-dependent calcium channel *CACNA1G* (FDR 7.75×10^{-69}) and was the most highly up-regulated (logFC 2.502). The *CACNA1G* gene is involved in the bone

morphogenetic protein (BMP) pathway, bone tissue mineralization, intracellular Ca signaling, and the Wnt β-catenin pathway.[20] BMP and Wnt β-catenin pathways are important for osteoblast differentiation and bone formation.[21–23] Depending on the form of alteration, changes to the BMP and Wnt β-catenin pathways can lead to bone fragility of different severities.[23–27] We surmise that differential expression of the *CACNA1G* gene might also reflect alterations in bone tissue metabolism.

In accordance with previous PMOP mRNA expression studies in circulating B cells, we found DEGs connected to the ERK/MAPK pathway. The estrogen receptor 1 (ESR1) and mitogen-activated protein kinase 3 (MAPK3) network has been proposed as a cause of increased osteoclastogenesis and decreased osteoblastogenesis.[28] However, the IPA analysis of the discovered PMOP profile from our study identified involvement of the DEGs in the FSH–ERK/MEK (MAPK) network, which is non-ESR dependent. Our findings support those of a previous study of PMOP in haploinsufficient FSH+/− mice, which showed activation of Gi2a-coupled FSH receptors stimulated MEK/Erk, NF-κβ, and Akt, and resulted in increased osteoclast activity and hypogonadal bone loss.[7] FSH-induced Gi2a, MEK/Erk, NF-κβ, and Akt signaling pathways are well-known osteoclast-stimulating pathways.[29] Recent investigations have also highlighted an FSH-dependent PMOP mechanism, caused by elevation of FSH and LH levels in elderly females.[5,6]

Although OP is connected with the aging process, the similar mean ages of the control (70.2) and OP patient (70.6) groups would likely exclude the possibility that the discovered mRNAs were a result of ''aging'' transcriptomes. We are confident that our results reflected a connection between the revealed candidate mRNA biomarkers and bone tissue reorganization. The matched BMI values of the groups also points to body weight being insignificant in terms of differences in the identified gene expression pattern of OP. The average BMD T-score of the control group for total hip (−0.19) and lumbar spine (0.26) showed high bone quality and allowed for more sensitive distinguishing of the contrasts between the mRNA expression patterns of OP patients and control subjects.

The reliability of our results might seem limited by the small number of study individuals, but the high power analysis value of 0.9 of the data should give cause for confidence in the soundness of our results. Furthermore, the transcriptome analysis power of 12 samples is 0.7 and 0.8 for both weak and strong compounds, respectively, which is sufficient to reveal differentially expressed genes.[30]

Blood cells do not express all bone cell proteins; thus, some protein translation changes may have gone unnoticed during our study. Nonetheless, the present study revealed a connection between whole blood mRNAs and FSH, and postmenopausal bone loss in humans.

Conclusion

In our study, we investigated whole transcriptome RNA sequencing of the blood serum of postmenopausal osteoporotic Estonian females, with the aim of revealing a candidate mRNA biomarker pattern for OP. We discovered a pattern of differently expressed mRNAs of OP that consisted of six genes: *CACNA1G*, *ALG13*, *SBK1*, *GGT7*, *MBNL3*, and *RIOK3*. This transcriptional landscape was connected to FSH-induced Gi2a, MEK/Erk, NF-κβ, and Akt signaling pathways, which are known to directly activate osteoclastogenesis and stimulate postmenopausal bone loss. The current findings may be useful for the development of a blood mRNA PMOP biomarker set which is a promising method of PMOP diagnosis and follow-up. Further studies with larger numbers of independent cohorts of PMOP patients and controls are required.

Authors' contribution: Study design: KM, SK, AM, OL. Study conduct: KM, OL, ER, EP, LZ. Data collection: KM, OL. Data analysis: KM, OL, LZ, SK. Data interpretation: KM, LZ, SK. Drafting the manuscript: KM, OL, LZ, SK, AM. Revising the manuscript content: KM, OL, LZ, EP, ER, SK, AM. Approving the final version of the manuscript: KM, OL, LZ, EP, ER, SK, AM. KM, SK, OL take responsibility for the integrity of the data analysis.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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