Effect of insulin resistance on whole blood mRNA and microRNA expression affecting bone turnover

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Abstract

Objective: To evaluate the effect of insulin resistance in obesity on the expression in whole blood of mRNA and miRNA affecting bone homeostasis as well as to estimate the influence of oral glucose load (OGTT) on serum osteocalcin concentration in obese individuals with and without insulin resistance.

Design: Cross-sectional study.

Methods: Carboxylated (cOC), undercarboxylated (ucOC) and total osteocalcin were measured by ELISA in the serum of obese subjects with insulin resistance ($n = 41$) and obese subjects without insulin resistance ($n = 41$) (control group) during OGTT. Analysis of gene expression (microarray) and miRNAs (real-time PCR) was performed in venous blood (representative samples) collected before OGTT from obese with insulin resistance and controls.

Results: Obese subjects with insulin resistance (higher HOMA-IR and lower oral glucose insulin sensitivity index) presented significantly increased expression of WNT signalling inhibitors (*DKK1*, *DKK2*, *SOST*, *SFRP1*) and downregulation of the key factor in WNT signalling – β catenin participating in osteoblasts differentiation. Expression of miRNA involved in osteoblastogenesis was also inhibited (miR-29b, miR-181a, miR-210, miR-324-3p). During OGTT, contrary to the control group, subjects with insulin resistance presented suppression of cOC and total OC decrease after 1 and 2 h of oral glucose load.

Conclusions: Obese subjects with insulin resistance may have defects in osteoblastogenesis that was demonstrated via key signalling molecules mRNA downregulation, and increased expression of WNT antagonists as well as inhibition of expression of miRNA participating in the regulation of osteoblast differentiation. Disturbed osteoblastogenesis in insulin-resistant subjects results in the suppression of blood carboxylated and total osteocalcin decrease during OGTT.

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Introduction

Inappropriate secretion of insulin and insulin resistance as well as chronic low-grade inflammation in obesity leads to disturbances in bone turnover. Ferron *et al.* reported that knockout of the insulin receptor on osteoblasts led to low bone mass in mice (1). By secreting osteocalcin (OC) osteoblasts participate in bone formation as well as energy metabolism. Osteocalcin in carboxylated form (cOC) interacts with hydroxyapatite crystals of the bone

matrix (2, 3). During bone resorption, undercarboxylated osteocalcin (ucOC) is released and participates in glucose and lipid metabolism (4, 5). Studies in mice showed that knockout of the osteocalcin gene (*Ocn*^{−/−}) led to obesity, increased serum triglycerides as well as suppression of insulin secretion and glucose intolerance (6). Human studies also showed that obese subjects with pre-diabetes had decreased ucOC blood levels, which

was inversely associated with HOMA-IR and serum insulin (7, 8).

Recent studies focused on the circulatory component of bone remodelling (9, 10, 11, 12), which appears to be tightly controlled by osteoblasts, osteocytes, immune cells, and the endocrine system. Namely, as regards bone formation, studies report that mesenchymal stem cells (MSC) from which osteoblasts are differentiated could be obtained not only from bone marrow but also from peripheral blood (9). It has been documented that osteoblast-lineage cells were present in the circulation of adult subjects and were associated with biochemical markers of bone formation (10). Circulating monocytes in turn could be considered cell cycle-arrested quiescent osteoclast precursors, which start their differentiation in haematopoietic tissues, afterwards circulate in the bloodstream, and then migrate to bones for final osteoclastogenesis (11, 12, 13, 14, 15). Taking into consideration the circulatory component of the process of bone formation and resorption, gene expression patterns in whole blood could partially reflect bone turnover processes in healthy as well as disease states.

In our study we concentrated on the effect of insulin resistance and disturbed oral glucose insulin sensitivity on the expression of mRNA and miRNA affecting bone homeostasis in whole blood from obese individuals as well as the influence of oral glucose load on serum osteocalcin concentration in obesity. We hypothesise that due to insulin resistance, the expression of mRNA and miRNA affecting bone turnover could be disturbed and consequently, blood osteocalcin concentration after OGTT could differ in comparison to subjects without insulin resistance.

Subjects and methods

Subjects

The cross-sectional study was performed in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and was approved by the Bioethics Committee of the Jagiellonian University in Cracow, Poland (opinion number KBET/82/B/2009). Study participants were recruited from patients at the Out-patient Clinic of Obesity and Lipid Disorder Treatment at the Department of Biochemistry, Jagiellonian University Medical College in Cracow, Poland from September 2009 till July 2013. Prior to participation in the study written consent was obtained from each subject after full explanation of the purpose and nature of procedures

used. Overweight and obese ($25 \leq \text{BMI} < 40 \text{ kg/m}^2$) women and men, aged 25–65 years were included into the study. Subjects suffering from chronic diseases (cardiovascular diseases, cancer, and chronic inflammation), diabetes mellitus and other metabolic disorders, impaired kidney or liver function were excluded from the study. Of the 195 participants recruited for the study, 50 declined to participate and 63 subjects did not meet the inclusion criteria. Thus, the study included 82 participants. Subjects included in the study were divided into two groups depending on oral glucose insulin sensitivity index (OGIS) (16): subjects without insulin resistance (control group with OGIS values $> 392 \text{ mL} \cdot \text{min}^{-1} \cdot \text{m}^{-2}$, $n=41$) and the group with insulin resistance (OGIS values $\leq 392 \text{ mL} \cdot \text{min}^{-1} \cdot \text{m}^{-2}$, $n=41$). All subjects underwent anthropometrical measurements: body mass index (BMI), waist-to-hip ratio (WHR) and body composition estimated with the bioelectrical impedance method using the Segmental Body Composition Analyser TANITA (Tanita, Tokyo, Japan).

Oral glucose tolerance test

Participants underwent a 2-h oral glucose tolerance test (OGTT) conducted according to the World Health Organization (WHO) and International Diabetes Federation (IDF) guidelines. Tests were performed in the morning hours (8:00–10:00h) after 10-h overnight fasting. Blood samples were collected at baseline (fasting), 60, and 120 min after the ingestion of 75 g glucose dissolved in 250 mL of water. All patients were asked not to drink alcohol or drinks containing caffeine for 3 days before the test.

Biochemical tests

Serum from blood samples collected before the OGTT was used for biochemical tests: total cholesterol, HDL cholesterol and inflammatory markers (CRP, IL-6). Total and HDL cholesterol were measured by enzymatic colorimetric methods using the MaxMat Analyzer (Maxmat SA, Montpellier, France). LDL cholesterol was calculated using the Friedewald formula. IL-6 was measured using ELISA (R&D Systems Europe, Ltd). CRP was measured by immunoturbidimetric method (APTEC Diagnostics nv, Sint-Niklaas, Belgium). In blood samples collected during the OGTT glucose, insulin, non-esterified free fatty acids (NEFAs), triglycerides (TGs), cOC and ucOC were measured. Serum glucose and triglycerides were measured by enzymatic colorimetric methods on

the MaxMat Analyzer. Serum insulin was measured by immunoradiometric method (DIAsource, ImmunoAssays, Louvain-la-Neuve, Belgium) using the gamma counter (LKB Instruments, Mount Waverley, Australia). Basal insulin resistance was estimated using homeostasis model of assessment (HOMA-IR) (17). To assess post oral glucose load insulin sensitivity, an oral glucose insulin sensitivity index (OGIS) proposed by Mari *et al.* (16) was used. OGIS was computed using a calculator for Excel spread sheet available on the website <http://webmet.pd.cnr.it/ogis>. Serum cOC and ucOC were determined by ELISA (Takara, Shiba, Japan). Total osteocalcin level was calculated as the sum of cOC and ucOC. NEFAs concentration was measured immediately in non-frozen plasma by enzymatic quantitative colorimetric method (Roche Diagnostics GmbH).

Sample collection, RNA and miRNA isolation

For analysis of gene expression and miRNAs, venous blood before OGTT (fasting sample) was collected into PAXgene Blood RNA Tubes (Becton Dickinson), following a 2-h room temperature incubation, samples were frozen at -80°C . Total RNA, miRNA enriched, was isolated using the PAXgene 96 Blood RNA Kit and GeneMATRIX Universal RNA/miRNA Purification Kit. RNA and miRNA quality were assessed in an Agilent Bioanalyzer 2100 using the RNA 6000 Nano kit (Agilent Technologies) and quantified by spectrophotometry using a NanoDrop ND-1000 UV-VIS spectrophotometer (Thermo Fisher Scientific). Samples with RIN > 7.0 were selected for further analysis.

Quantification of mRNA transcripts

Gene expression profiles were determined using Single Color 39494 human oligonucleotide arrays and reagents from Agilent Technologies. Each separate RNA sample was hybridised to a single array. Labelling of 100 ng of total RNA was done using the Quick Amp labelling kit according to the manufacturer's protocol. RNA concentration and dye incorporation were measured using Nanodrop ND-1000. Signal intensity of the labelled cRNA was measured by hybridisation to a human gene microarray. Detection of the fluorescence signal was performed by the Agilent SureScan Microarray Scanner using extraction software (Agilent Technologies). For microarray hybridisation, nine samples were used (four samples from the control group, five samples from obese subjects with insulin resistance). Microarray data analysis was performed using Gene Spring version 13 (Agilent Technologies). To identify

differentially expressed genes each data set of fluorescence signals was normalised using median shift algorithm (shifted to 75 percentile) with background correction to the median of all samples. Normalised signal was used for fold of change (FC) calculation. FC was calculated in relation to obese with insulin resistance versus obese without insulin resistance and used as expression level.

Quantification of microRNA (miRNA, miR) transcripts by TLDA

For relative quantification of miRNA by Real-time PCR, TaqMan Low-Density Array (TLDA) was used (Thermo Scientific). Reverse transcription was performed with TaqMan MicroRNA Reverse Transcription Kit and MegaPlex Human Pool A RT primers. Preamplification was enabled by Megaplex™ PreAmp Kit and Primers. TaqMan® Array Human MicroRNA A+B Cards Set v3.0 were employed for the accurate quantitation of 754 human microRNA by real-time PCR. The arrays were run in 7900HT Fast Real-Time PCR system (Thermo Scientific). Relative miRNA levels were calculated using DataAssist v 3.01 tool and expressed as $\text{RQ} = 2^{-(\Delta\text{Ct})_{\text{obese with insulin resistance}} / 2^{-(\Delta\text{Ct})_{\text{obese without insulin resistance}}}$. RNU44, RNU48 and U6 snRNA were used as endogenous controls.

Statistical analysis

Analyses of nominal data were performed using chi-square (χ^2) test. The Shapiro–Wilk test was used to analyse data for a Gaussian distribution. For verification of homogeneity of variance Levene's test was used. Continuous variables were log transformed if required. Normally distributed data are presented as mean $\pm$ S.E.M. otherwise as median and lower-upper quartile range (25–75%). Differences between obese with insulin resistance versus obese without insulin resistance were calculated by unpaired *t*-test or Mann Whitney *U* test (for non-normally distributed data). Potential differences in serum glucose, insulin, NEFAs, triglycerides and osteocalcins during OGTT were calculated with repeated-measures ANOVA. Values at single time points were compared by Tukey *post hoc* test. *P* values less than 0.05 were considered significant. Statistical analyses were performed with the Statistical software (StatSoft Polska, Krakow, Poland). The list of significantly regulated transcripts in whole blood of obese with impaired insulin sensitivity versus obese without insulin resistance with $P < 0.05$ was generated using statistical filtering (Moderated *T*-test with Benjamini–Hochberg multiple test correction).

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Analysis of regulated pathways was performed using PathVisio 3 software (<https://www.pathvisio.org>).

P value for differences in miRNA expression in whole blood between obese with insulin resistance versus obese without insulin resistance was calculated using *t*-test as comparison of the normalised $2^{(-\Delta Ct)}$ values of the two groups. The comparison of biochemical measurements as well as regulated transcripts and miRNA expression were not adjusted for confounders because obese with and without insulin resistance were not statistically different regarding possible confounders such as sex, age, BMI and adipose tissue mass.

Results

Characteristics of the subjects

The basal characteristics of participants is summarised in Table 1. Both groups of subjects (subjects with insulin

resistance $n=41$ vs individuals without insulin resistance $n=41$) did not differ significantly regarding age, sex, BMI, waist circumference, WHR and adipose tissue mass. However, in comparison to control group (subjects without insulin resistance), the insulin resistance subjects had higher fasting serum glucose (5.31 ± 0.08 vs 5.06 ± 0.08 mmol/L ($P=0.018$)) and higher HOMA-IR value (4.04 (2.41 – 5.46) vs 2.60 (2.15 – 3.67) ($P=0.027$)). During OGTT 1-h serum glucose was higher in the group of subjects with insulin resistance than controls (9.08 ± 0.38 vs 7.90 ± 0.39 mmol/L, $P=0.001$) (Fig. 1A). In these subjects neither fasting nor 2-h mean serum glucose was above the upper reference limit: 5.55 and 7.8 mmol/L, respectively. However, 12 subjects had elevated fasting serum glucose (5.6–6.4 mmol/L). Impaired glucose tolerance was observed in 9 individuals with 2-h serum glucose between 7.8 and 10.5 mmol/L. Subjects with insulin resistance had higher serum insulin in comparison to control group ($P=0.016$). Serum insulin increased 1 h after glucose load by about 500% and then gradually fell

Table 1 Basal characteristics of subjects included in the study.

	Obese with insulin resistance ($n = 41$)	Obese without insulin resistance ($n = 41$)	<i>P</i> *
Age (years)	$48.32 \pm 1.63^\dagger$	45.68 ± 1.88	0.292
Sex, female (%)	68	80	>0.05
BMI (kg/m ²)	33.10 (31.12 – 35.54) [‡]	32.08 (28.98 – 34.82)	0.124
WC (cm)			
Females	99 (92.8 – 106.5)	96 (90 – 101.5)	0.281
Males	115 (109.5 – 121)	114 (102 – 116)	0.237
WHR			
Females	0.84 (0.82 – 0.91)	0.82 (0.80 – 0.85)	0.113
Males	1.02 (0.99 – 1.04)	0.98 (0.95 – 0.99)	0.024
Adipose tissue mass (%)	38.60 (33.35 – 41.55)	38.80 (33.85 – 41.70)	0.954
Systolic BP (mm Hg)	130 (120 – 140)	122 (116 – 130)	0.298
Diastolic BP (mm Hg)	84 (80 – 90)	81 (76 – 90)	0.438
Total cholesterol (mmol/L)	5.49 ± 0.16	5.50 ± 0.17	0.945
HDL cholesterol (mmol/L)	1.27 ± 0.04	1.29 ± 0.03	0.646
LDL cholesterol (mmol/L)	3.50 ± 0.14	3.59 ± 0.16	0.670
NEFA (mmol/L)	0.72 ± 0.03	0.78 ± 0.04	0.432
Triglycerides (mmol/L)	1.69 ± 0.12	1.39 ± 0.11	0.049
Glucose (mmol/L)	5.31 ± 0.08	5.06 ± 0.08	0.018
Insulin (uIU/mL)	17.38 ± 1.40	14.50 ± 1.20	0.088
HOMA-IR	4.04 (2.41 – 5.46)	2.60 (2.15 – 3.67)	0.027
IL-6 (pg/mL)	1.62 ± 0.19	1.33 ± 0.12	0.224
CRP (mg/L)	2.18 (1.00 – 3.81)	1.53 (0.63 – 4.07)	0.919
Vitamin D (ng/mL)	13.24 (10.48 – 18.64)	16.71 (9.36 – 24.57)	0.207
ucOC (ng/mL)	3.57 ± 0.37	3.04 ± 0.28	0.446
cOC (ng/mL)	5.47 ± 0.29	6.21 ± 0.42	0.137
Total OC (ng/mL)	9.03 ± 0.52	9.25 ± 0.51	0.291

*Significant difference between obese with insulin resistance and obese without insulin resistance, (unpaired *t*-test or Mann–Whitney *U* test for non-normally distributed variables; nominal data were analysed by χ^2 test), $P < 0.05$. [†]Mean $\pm$ s.e.m.; all such values. [‡]Median (25–75%); all such values. BMI, body mass index; BP, blood pressure; cOC, carboxylated osteocalcin; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment; CRP, C-reactive protein; IL-6, interleukin 6; LDL, low-density lipoprotein; NEFA, non-esterified fatty acids; OC, osteocalcin; ucOC, undercarboxylated osteocalcin; WC, waist circumference; WHR, waist-to-hip ratio.

Figure 1

Blood levels of glucose (A), insulin (B), non-esterified fatty acids (NEFAs) (C) and triglycerides (D) during oral glucose tolerance test. Data are presented as means $\pm$ S.E.M. Statistical analyses were performed with repeated-measures ANOVA and denote differences between obese with insulin resistance and obese without insulin resistance participants (a), differences over time (b) and differences owing to interaction between group and time (ab). Values at single time points were compared by Tukey *post hoc* tests. Significant differences are indicated as follows: *** $P < 0.001$.

after 2 h by about 20% in the group with insulin resistance and approximately by 50% in the control group (Fig. 1B). There were no statistically significant differences in serum LDL, HDL and total cholesterol between studied groups except for serum triglycerides (1.69 ± 0.12 vs 1.39 ± 0.11 , $P = 0.049$).

As regards plasma NEFAs in response to glucose load, we found no statistically significant differences between the studied groups ($P = 0.309$). In both studied groups plasma NEFAs gradually decreased after 1 and 2 h of the test in comparison to baseline values ($P < 0.001$) (Fig. 1C). However, we found that during OGTT serum triglycerides concentration was higher in the group of subjects with insulin resistance than controls ($P = 0.034$) (Fig. 1D). We did not observe any statistically significant differences between the studied groups in serum inflammatory markers: IL-6 (1.62 ± 0.19 vs 1.33 ± 0.19 pg/mL, $P = 0.224$), CRP (2.18 (1.00–3.81) vs 1.53 (0.63–4.07) mg/L, $P = 0.919$), which probably was connected with similar BMI, WHR and adipose tissue mass in both groups of patients (Table 1).

Figure 2

Blood concentrations of carboxylated osteocalcin (cOC) (A), undercarboxylated osteocalcin (ucOC) (B) and total osteocalcin (C) during oral glucose tolerance test. Data are presented as means $\pm$ S.E.M. Statistical analyses were performed with repeated-measures ANOVA and denote differences between obese with insulin resistance and obese without insulin resistance participants (a), differences over time (b) and differences owing to interaction between group and time (ab). Values at single time points were compared by Tukey *post hoc* tests. Significant differences are indicated as follows:

* $P < 0.05$, ** $P < 0.01$.

Effects of insulin resistance in obesity on serum osteocalcin during OGTT

Although baseline serum osteocalcin (cOC) was similar in both studied groups (5.47 ± 0.19 vs 6.21 ± 0.19 ng/mL, $P = 0.139$) (Table 1), the response to oral glucose load was different in respect to serum cOC ($P = 0.028$) (Fig. 2A). Surprisingly, in subjects without insulin resistance serum cOC gradually decreased after 1 h (5.41 ± 0.32 vs 6.21 ± 0.19 ng/mL, $P < 0.05$) and 2 h (5.06 ± 0.32 vs 6.21 ± 0.19 ng/mL, $P < 0.01$) of OGTT compared to baseline values. This effect was suppressed in subjects with insulin resistance. Similar to serum cOC, total osteocalcin concentration in control group but not in subjects with insulin resistance ($P = 0.041$) was gradually reduced at 1 h (8.17 ± 0.44 vs 9.25 ± 0.44 ng/mL, $P < 0.01$) and 2 h (7.53 ± 0.43 vs 9.25 ± 0.44 ng/mL, $P < 0.01$) after glucose load (Fig. 2C). However, no statistically significant differences were observed in serum ucOC during OGTT

between the studied groups. In comparison to baseline values, both groups of subjects presented gradually reduced serum ucOC levels during 1 h and 2 h of OGTT ($P < 0.001$) (Fig. 2B).

Effects of insulin resistance in obesity on the expression of mRNA and miRNA participating in the regulation of osteoblasts and osteoclasts differentiation

Results of gene expression analysis in whole blood of obese subjects with insulin resistance are presented in Table 2. Analysis of regulated pathways performed by PathVisio 3 software revealed that two groups of studied subjects differ regarding following pathways: osteoclast signalling, osteoblast signalling, Wnt Signalling pathway, vitamin D receptor pathway, TGF β signalling pathway, insulin signalling, cytokines and inflammatory response.

Obese subjects with insulin resistance in comparison to control group presented inhibition of insulin signalling pathway genes expression in whole blood. In comparison to subjects without insulin resistance, they had downregulation of receptors for IGF1 (*IGF1R*), P13-Kinase Isoforms (*PIK3CD*, *PIK3CG*, *PIK3C3*, *PIK3R1*, *PIK3R2*), genes participating in PDK/AKT signalling pathway (*PDPK1*, *AKT2*, *GSK3A*, *GSK3B*) as well as transcription factor *FOXO1*. As regards cytokines and inflammatory response, we found that expression of genes coding for cytokines/antigens taking part in inflammatory response was upregulated: *IL6*, *IL12B*, *IL12A*. The mRNA levels of genes involved in the regulation of chemotaxis were increased in subjects with insulin resistance versus individuals without insulin resistance: *CCL2*, *CXCL8*, *CXCL10*, *CXCL9*, *CXCL11*. Expression of other pro-inflammatory cytokines was also upregulated: *IFNA10*, *IFNA21*, *IFNA14*, *IFNB1*, *IFNA16*, *IFNA6*, *IFNA7*, *IFNB1*. Analysis of gene expression in subjects with insulin resistance also revealed upregulation of genes coding for *CRP*, *CSF1* (MCP1), receptor for IL-1 (*IL1R1*) as well as co-stimulatory molecules: *CD40*, *CD80*.

The mRNA levels of genes responsible for osteoblasts signalling differentiation and maturation were also differentially regulated in obese with insulin resistance in comparison to subjects without insulin resistance. The expression in whole blood of genes being early markers of osteoblasts differentiation was statistically significantly regulated in obese subjects. We found downregulation of alkaline phosphatase (*ALPL*) and upregulation of osteopontin (*SPP1*), bone sialoprotein (*IBSP*), type 1 alpha collagen (*COL1A1*) in obese subjects with insulin

resistance. The expression of osteocalcin gene (*BGLAP*) was unchanged (data not shown). Among factors which modulate osteoblast differentiation, we observed downregulation of *TGFB1*, receptor for vitamin D (*VDR*), but mRNA for bone morphogenic proteins (*BMP2*, *BMP4*, *BMP7*) and the receptor for PTH (*PTH1R*) were upregulated in insulin resistance subjects. Analysis of gene expression also revealed upregulation of BMP antagonist *GREM2* and *TGFB3* as well as inhibition of the receptor for TGF β (*TGFB2*) in obese subjects with insulin resistance. However, the expression of Wnt signalling antagonists: *DKK1*, *DKK2*, *DKK4*, sclerostin (*SOST*), *SFRP1*, *DUSP2*, *ACVR2A* and *WIF1* was upregulated. We also found upregulation of Wnt signalling agonists *WNT3A*, *WNT5A* and *FZD* and their receptor *LRP5*. However, *CTNNB1* and its nuclear partners *LEF-1/TCF4* transcription factor family were downregulated in obese subjects with insulin resistance. Expression of key transcription factors in osteoblast differentiation (*RUNX2*, osterix *SP7*, *MSX2*, *DLX5*, *TWIST1* and *TWIST2*) was upregulated. However, expression of *RUNX2* degradation protein gene (*SMURF2*) was upregulated. Expression of another osteoblast differentiation factor *ATF4* was inhibited in whole blood of subjects with insulin resistance as compared to subjects without insulin resistance.

The mRNA levels of genes participating in osteoclasts signalling differentiation were statistically significantly upregulated in obese subjects with insulin resistance. As regards RANKL/RANK/OPG pathway, we found upregulated expression of *TNFSF11A* (RANK), *TNFRSF11B* (Osteoprotegerin) and *TNFSF11* (RANKL). However, expression ratio of *TNFSF11/TNFRSF11B* was 1. Although gene expression of *CSF1* (MCP1) was increased, we observed inhibition of *SPI1* (PU.1). Moreover, gene expression of *NEATC1* as well as regulated osteocytes' genes, such as tartrate-resistant acid phosphatase (*ACP5* (TRAP), *ITGA5*), was downregulated.

Expression of miRNAs participating in regulation of osteoblasts and osteoclasts differentiation is shown in Table 3. We found significant downregulation of miR-324-3p in obese subjects with insulin resistance compared to volunteers without insulin resistance. It was demonstrated that miR-324-3p expression is inhibited in subjects with type 2 diabetes and is associated with disturbances in bone microstructure (18, 19). In blood of obese subjects with insulin resistance, miR-181a expression was also downregulated, which could negatively affect osteoblastic differentiation due to upregulation of BMP antagonist expression (*GREM2*). Contrary to control group, insulin-resistant subjects had decreased expression

Table 2 The differentially expressed genes of whole blood involved in pathways related to bone remodelling in obese subjects with insulin resistance. Significantly regulated transcripts (NCBI symbols) with $P < 0.05$ in whole blood of obese with insulin resistance versus obese without insulin resistance was generated using Moderated T -test with Benjamini-Hochberg multiple test correction.

Gene symbol	Gene name	FC	P (corr)	Ensembl ID	Entrez Gene ID	GenBank accession	Ref Seq accession
Insulin signalling pathway							
AKT2	v-Akt murine thymoma viral oncogene homolog 2	-2.75	0.021	ENST00000476266	208	NM_001626	NM_001626
FOXO1	Forkhead box O1	-2.94	0.019	ENST00000379561	2308	NM_002015	NM_002015
GSK3A	Glycogen synthase kinase 3 alpha	-2.03	0.021	ENST00000222330	2931	NM_019884	NM_019884
GSK3B	Glycogen synthase kinase 3 beta	-2.44	0.021	ENST00000473886	2932	NM_002093	NM_002093
IGF1R	Insulin-like growth factor 1 receptor	-2.72	0.021	ENST00000268035	3480	NM_000875	NM_000875
PDPK1	3-Phosphoinositide dependent protein kinase 1	-2.29	0.023	ENST00000342085	5170	NM_002613	NM_002613
PIK3C3	Phosphatidylinositol 3-kinase catalytic subunit type 3	-2.10	0.036	ENST00000587328	5289	NM_002647	NM_002647
PIK3CD	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta	-2.10	0.022	ENST00000377346	5293	NM_005026	NM_005026
PIK3CG	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma	-2.33	0.045	ENST00000496166	5294	NM_002649	NM_002649
PIK3R1	Phosphoinositide-3-kinase regulatory subunit 1 (alpha)	-2.60	0.021	ENST00000521381	5295	NM_181523	NM_181523
PIK3R2	Phosphoinositide-3-kinase regulatory subunit 2 (beta)	-2.14	0.021	ENST0000022254	5296	NM_005027	NM_005027
Inflammatory molecules pathways							
CCL2	Chemokine (C-C motif) ligand 2	2.31	0.021	ENST00000582017	6347	NM_002982	NM_002982
CRP	C-reactive protein pentraxin-related	2.44	0.021	ENST00000368111	1401	NM_000567	NM_000567
CSF1	Colony-stimulating factor 1 (macrophage)	2.38	0.021	ENST00000369801	1435	NM_172210	NM_172210
CD40	CD40 molecule. TNF receptor superfamily member 5	1.76	0.040	ENST00000477696	958	NM_001250	NM_001250
CD80	CD80 molecule	2.68	0.035	ENST00000264246	941	NM_005191	NM_005191
CXCL8	Chemokine (C-X-C motif) ligand 8	2.88	0.021	ENST00000307407	3576	NM_000584	NM_000584
CXCL9	Chemokine (C-X-C motif) ligand 9	2.24	0.030	ENST00000264888	4283	NM_002416	NM_002416
CXCL10	Chemokine (C-X-C motif) ligand 10	2.02	0.034	ENST00000306602	3627	NM_001565	NM_001565
CXCL11	Chemokine (C-X-C motif) ligand 11	2.23	0.021	ENST00000306621	6373	NM_005409	NM_005409
IFNA6	Interferon alpha 6	2.22	0.021	ENST00000259555	3443	NM_021002	NM_021002
IFNA7	Interferon alpha 7	2.50	0.048	ENST00000239347	3444	NM_021057	NM_021057
IFNA10	Interferon alpha 10	2.26	0.021	ENST00000357374	3446	NM_002171	NM_002171
IFNA14	Interferon alpha 14	2.08	0.031	ENST00000380222	3448	NM_002172	NM_002172
IFNA16	Interferon alpha 16	1.99	0.036	ENST00000380216	3449	NM_002173	NM_002173
IFNA21	Interferon alpha 21	2.19	0.021	ENST00000380225	3452	NM_002175	NM_002175
IFNB1	Interferon beta 1 fibroblast	2.16	0.021	ENST00000380232	3456	NM_002176	NM_002176
IFNB1	Interferon beta 1 fibroblast	2.16	0.021	ENST00000380232	3456	NM_002176	NM_002176
IL1R1	Interleukin 1 receptor type 1	2.02	0.024	ENST00000413623	3554	NM_000877	NM_000877
IL6	Interleukin 6	3.99	0.021	ENST00000406575	3569	NM_005249745	NM_005249745
IL12A	Interleukin 12A	1.99	0.048	ENST00000496308	3592	NM_000882	NM_000882
IL12B	Interleukin 12B	2.19	0.021	ENST00000231228	3593	NM_002187	NM_002187
Osteoblasts differentiation pathways							
ALPL	Alkaline phosphatase liver/bone/kidney	-2.27	0.029	ENST00000374832	249	NM_000478	NM_000478
ATF4	Activating transcription factor 4	-2.38	0.021	ENST00000337304	468	NM_001675	NM_001675
ACVR2A	Activin A receptor type IIA	2.18	0.021	ENST00000495775	92	NM_001616	NM_001616
BMP2	Bone morphogenetic protein 2	2.34	0.021	ENST00000378827	650	NM_001200	NM_001200
BMP4	Bone morphogenetic protein 4	2.25	0.021	ENST00000558984	652	NM_001202	NM_001202
BMP7	Bone morphogenetic protein 7	2.26	0.021	ENST00000395863	655	NM_001719	NM_001719

(Continued)

Table 2 Continued.

Gene symbol	Gene name	FC	P (corr)	Ensembl ID	Entrez Gene ID	GenBank accession	Ref Seq accession
COL1A1	Collagen type I alpha 1	1.87	0.048	ENST00000225964	1277	NM_000088	NM_000088
CTNNA1	Catenin (cadherin-associated protein) beta 1 88kDa	-2.49	0.021	ENST00000396185	1499	NM_001904	NM_001904
DKK1	Dickkopf WNT signalling pathway inhibitor 1	2.10	0.022	ENST00000373970	22943	NM_012242	NM_012242
DKK2	Dickkopf WNT signalling pathway inhibitor 2	2.25	0.021	ENST00000285311	27123	NM_014421	NM_014421
DKK4	Dickkopf WNT signalling pathway inhibitor 4	2.48	0.025	ENST00000220812	27121	NM_014420	NM_014420
DLX5	Distal-less homeobox 5	2.18	0.021	ENST00000222598	1749	NM_005221	NM_005221
DUSP2	Dual specificity phosphatase 2	1.95	0.032	ENST00000488952	1844	NM_004418	NM_004418
FZD3	Frizzled class receptor 3	2.28	0.040	ENST00000537916	7976	NM_017412	NM_017412
FZD4	Frizzled class receptor 4	2.23	0.021	ENST00000531380	8322	NM_012193	NM_012193
FZD5	Frizzled class receptor 5	2.27	0.021	ENST00000295417	7855	NM_003468	NM_003468
FZD6	Frizzled class receptor 6	2.50	0.022	ENST00000522566	8323	NM_003506	NM_003506
FZD7	Frizzled class receptor 7	2.21	0.021	ENST00000286201	8324	NM_003507	NM_003507
FZD8	Frizzled class receptor 8	2.17	0.021	ENST00000374694	8325	NM_031866	NM_031866
FZD9	Frizzled class receptor 9	2.60	0.021	ENST00000344575	8326	NM_003508	NM_003508
FZD10	Frizzled class receptor 10	2.22	0.021	ENST00000229030	11211	NM_007197	NM_007197
GREM2	Gremelin 2; DAN family BMP antagonist	5.08	0.021	ENST00000318160	64388	NM_022469	NM_022469
IBSP	Integrin-binding sialoprotein	2.20	0.021	ENST00000226284	3381	NM_004967	NM_004967
LEF1	Lymphoid enhancer-binding factor 1	-2.64	0.021	ENST00000503879	51176	NM_016269	NM_016269
LRP5	Low-density lipoprotein receptor-related protein 5	3.02	0.021	ENST00000529993	4041	NM_002335	NM_002335
MSX2	msh homeobox 2	2.30	0.021	ENST00000239243	4488	NM_002449	NM_002449
PTH1R	Parathyroid hormone 1 receptor	2.18	0.021	ENST00000427125	5745	NM_000316	NM_000316
RUNX2	Runt-related transcription factor 2	2.11	0.031	ENST00000371436	860	NM_001015051	NM_001015051
SFRP1	Secreted frizzled-related protein 1	2.29	0.021	ENST00000220772	6422	NM_003012	NM_003012
SMAD4	SMAD family member 4	-4.07	0.021	ENST00000342988	4089	NM_005359	NM_005359
SMURF2	SMAD specific E3 ubiquitin protein ligase 2	2.26	0.021	ENST00000582081	64750	NM_022739	NM_022739
SOST	Sclerostin	2.19	0.021	ENST00000301691	50964	NM_025237	NM_025237
SP7	Sp7 transcription factor	2.13	0.022	ENST00000303846	121340	NM_152860	NM_152860
SPP1	Secreted phosphoprotein 1	2.08	0.022	ENST00000360804	6696	NM_001040058	NM_001040058
TCF4	Transcription factor 4	-2.28	0.021	ENST00000570177	6925	NM_003199	NM_003199
TGFB1	Transforming growth factor beta 1	-2.61	0.021	ENST00000221930	7040	NM_000660	NM_000660
TGFB3	Transforming growth factor beta 3	2.21	0.021	ENST00000238682	7043	NM_003239	NM_003239
TGFB2	Transforming growth factor beta receptor II (70/80kDa)	-1.84	0.048	ENST00000295754	7048	NM_001024847	NM_001024847
TWIST1	Twist family bHLH transcription factor 1	3.05	0.021	ENST00000354571	7291	XR_428085	XR_428085
TWIST2	Twist family bHLH transcription factor 2	2.24	0.021	ENST00000448943	117581	NM_057179	NM_057179
VDR	Vitamin D (1,25- dihydroxyvitamin D3) receptor	-2.04	0.026	ENST00000229022	7421	NM_001017535	NM_001017535
WIF1	WNT inhibitory factor 1	2.27	0.023	ENST00000286574	11197	NM_007191	NM_007191
WNT3A	Wingless-type MMTV integration site family, member 3A	2.26	0.021	ENST00000284523	89780	NM_033131	NM_033131
WNT5A	Wingless-type MMTV integration site family, member 5A	2.26	0.021	ENST00000264634	7474	NM_003392	NM_003392
Osteoclasts differentiation pathways							
ACP5	Acid phosphatase 5 tartrate resistant (TRAP)	-1.80	0.040	ENST00000592828	54	NM_001611	NM_001611
ITGA5	Integrin alpha 5 (fibronectin receptor alpha polypeptide)	-2.41	0.021	ENST00000552564	3678	NM_002205	NM_002205
NFATC1	Nuclear factor of activated T-cells cytoplasmic calcineurin-dependent 1	-2.30	0.021	ENST00000329101	4772	NM_172387	NM_172387
SPI1	Spi-1 proto-oncogene	-2.40	0.021	ENST00000227163	6688	NM_001080547	NM_001080547
TNFRSF11A	Tumour necrosis factor receptor superfamily member 11a. NFKB activator	2.27	0.021	ENST00000616710	8792	NM_003839	NM_003839
TNFRSF11B	Tumour necrosis factor receptor superfamily member 11b	2.13	0.021	ENST00000521597	4982	NM_002546	NM_002546
TNFSF11	Tumour necrosis factor (ligand) superfamily member 11	2.10	0.027	ENST00000358545	8600	NM_033012	NM_033012

Table 3 Relative quantification (RQ) of miRNA expression profile in obese subjects with insulin resistance versus obese without insulin resistance individuals.

miRNA	RQ	P value
hsa-miR-29b	0.474	0.046
hsa-miR-181a	0.297	0.009
hsa-miR-210	0.458	0.035
hsa-miR-324-3p	0.467	0.012

P value for differences in miRNA expression in whole blood between obese with insulin resistance and obese without insulin resistance subjects was calculated using Student's *t*-test as comparison of the normalised $2^{-(\Delta\Delta Ct)}$ values of the two groups.

of miR-210 in whole blood, which can indicate that osteoblast proliferation and differentiation were inhibited. Moreover, we found decreased miR-29b expression, which could contribute to osteogenesis disturbances due to increased expression of osteoblast differentiation inhibitors (*TGFB3*, *ACVR2A*, *DUSP2*) (20).

Discussion

The study is the first one focusing on both mRNA and microRNA in whole blood, associated with bone remodelling in obese subjects with and without insulin resistance. Analysis of gene expression was performed in whole blood. It is well known that RNA information from whole blood resides in circulating blood leukocytes. However, when analysis of gene expression connected with bone turnover is performed in whole blood, circulatory component of bone remodelling should be taken into consideration. Namely, osteoblast-lineage cells circulate in peripheral blood in physiologically significant numbers which correlate with markers of bone formation (10). Osteoclast precursors are also detectable in the peripheral circulation (11).

In comparison to the control group (subjects without insulin resistance), the study group had a higher degree of insulin resistance determined by lower OGIS and higher HOMA-IR values. Studied subjects with insulin resistance had no diabetes but 12 of 41 subjects (29%) had impaired fasting glucose (5.6–6.4 mmol/L) and nine subjects (22%) had impaired glucose tolerance (2-h serum glucose 7.8–10.5 mmol/L), together 17 subjects (41%) had pre-diabetes. Obese insulin-resistant subjects had higher basal and post-glucose load serum insulin, glucose and triglycerides in comparison to the control group. Analysis of gene expression in whole blood from obese subjects with insulin resistance confirmed insulin signalling pathway defects. We observed downregulation of *IGF1R*,

P13-kinase isoforms, genes participating in PDK/AKT signalling pathway. It should be stressed that both groups did not differ regarding BMI, adipose tissue mass as well as serum inflammatory markers.

Some studies reported that osteoblasts from obese insulin resistant subjects were less active and produced less collagen as well as had lower bone formation index defined as quotient of procollagen type 1 amino-terminal propeptide to bone alkaline phosphatase (PINP/BAP) (21). In our studies we also reported that disturbances in insulin signalling could affect osteoblastogenesis by inhibiting expression of genes connected with Wnt, BMP signalling pathway and upregulation of genes participating in inflammatory response. Indeed, we found downregulation of β -catenin (*CTNNB1*), which via mediating WNT signal transduction, plays a key role in the commitment of early progenitors to osteoblast precursors. Although we observed upregulation of WNT signalling pathway agonists (*WNT3A*, *WNT5A* and *FZD*) as well as their receptor *LRP5*, the expression of this pathway antagonists was also upregulated (*SFRP*, *WIF-1*, *SOST*, *DKK1*, *DKK2*). WNT ligands can interact with antagonists, including (SFRP) family and WNT inhibitory factor 1 (WIF1), preventing activation of the pathway. In turn *LRP5/6* co-receptor activity is inhibited by *SOST* (sclerosteosis gene product) (22) and *Dickkopf* (*DKK*) families (23, 24), all of which bind *LRP5/6*, that in consequence leads to the downregulation of β catenin gene expression. Although we observed the upregulation of gene expression for BMPs promoting bone formation, such as *BMP2*, *BMP4*, *BMP6* (25), the gene expression of *SMAD4* in BMP signalling was downregulated in subjects with insulin resistance. It has been reported previously that conditional deletion of *SMAD4* in osteoblasts, leads to lower bone mineral density, decreased bone volume, decreased bone formation rate, and a reduced number of osteoblasts (26).

Although gene expression of key transcription factor in osteoblast differentiation – *RUNX2* was upregulated in obese subjects with insulin resistance, its protein concentration could be decreased by upregulation of *SMURF2* (27) as well as *TWIST1* and *TWIST2* (28). Bialek *et al.* showed that *RUNX2*-induced osteoblast gene expression occurs only when expression of *TWIST* genes disappears in osteoblast precursors (29). *TWIST* proteins' antiosteogenic function is mediated by a *TWIST* box, which interacts with the *RUNX2* DNA-binding domain to inhibit its function.

Transcriptional activity of *RUNX2* might also be regulated by $1\alpha,25$ -dihydroxy vitamin D3 through *VDR*.

It was reported previously that *RUNX2* and *VDR* are co-expressed in developing skeletal elements, and interaction between *VDR* and *RUNX2* stabilises transcriptional complexes at specific promoters and induces osteoblast-specific genes expression such as osteocalcin (*Ocn*) (30).

In our study, both groups of subjects had low serum vitamin D concentration (below target range: 30–80 ng/mL), and there were no statistically significant differences between studied groups. However, a decrease in *VDR* gene expression was observed in the group with insulin resistance in comparison to the control group, which could in consequence affect bone remodelling. Our data showed that osteocalcin gene expression was not regulated, which could be explained by the fact that osteocalcin is a late marker of osteoblasts differentiation (31). However, the expression of some markers of cell differentiation towards osteoblasts as osteopontin, bone sialoprotein and collagen were upregulated in subjects with insulin resistance, which may confirm the presence of osteoprogenitors in the circulation.

Obese subjects with insulin resistance contrary to subjects without insulin resistance, responded to glucose load with a suppressed decrease in serum bone formation markers: total and carboxylated osteocalcin. Such data are in agreement with recently published results indicating that in obese subjects the decrease in serum cOC was suppressed in comparison to non-obese volunteers after oral glucose load (32, 33). Taking into consideration the fact that cOC accumulates mainly in bone (only 15% is released into circulation) its decrease during OGTT in blood of subjects without insulin resistance could suggest increased bone formation. However, we did not observe suppression of ucOC decrease in obese subjects with insulin resistance in comparison to the control group as was reported in the study by Schwetz (34). On the other hand, Pollock *et al.* reported that in humans, cOC may influence insulin sensitivity, whereas ucOC could affect β -cell function and seems to be important in subjects with pre-diabetes and diabetes (35).

We postulate that after nutrients ingestion an increase in inflammatory response and insulin resistance could contribute to bone turnover alterations. Although we did not observe increases in pro-inflammatory cytokines on protein levels in serum at fasting, expression of genes taking part in inflammatory response that is *CXCL8*, *CXCL10*, *CXCL9*, *CXCL11* was upregulated in subjects with insulin resistance in comparison to subjects without insulin resistance. Such gene expression patterns could influence osteoblasts differentiation. *In vitro* studies

reported that pro-inflammatory cytokines such as tumour necrosis factor α (TNF α) suppressed osteoblast maturation by inhibiting the expression of *RUNX2* (36). Pro-inflammatory cytokines disrupt the WNT signalling pathway. Resolution of inflammation was accompanied by striking changes in the expression of regulators of the Wnt/ β -catenin pathway (37).

Data from previous studies revealed that pro-inflammatory cytokines can stimulate osteoclastogenesis by modulating the RANK/RANKL/OPG system (27). Binding RANKL to its receptor, RANK, on the surface of osteoclasts and their precursors regulates the differentiation of precursors into multinuclear osteoclasts and osteoclast activation. Osteoprotegerin (OPG, TNFSF11) is secreted by osteoblasts and osteogenic stromal stem cells and protects the skeleton from excessive bone resorption by binding to RANKL and preventing it from interacting with RANK. The RANKL/OPG ratio is therefore a determinant of bone mass. Indeed, we found increased expression of *TNFSF11*, *TNFRSF11B* and *RANK* in obese subjects with insulin resistance. However, expression ratio *TNFSF11*/*TNRSF11B* was equal to 1, which suggests that osteoclastogenesis is not activated in these subjects. Moreover, *PU.1* (*SP11*) and the master regulator of osteoclast differentiation – nuclear factor of activated T-cells cytoplasmic 1 (*NEATC1*), which regulate a number of osteoclast-specific genes, were downregulated in obese subjects with insulin resistance (38). We also observed that expression of osteoclast-specific genes including *ACP5* (*TRAP*), *ITGA5* was downregulated. These results suggest that insulin resistance in obesity leads to inhibition of osteoblastogenesis but does not stimulate osteoclastogenesis and bone resorption.

Analysis of miRNA expression in whole blood of obese subjects with insulin resistance appears to confirm gene expression patterns. MicroRNAs (miRs/miRNAs) are endogenous small, non-coding RNAs that positively or negatively regulate gene expression and cellular processes via the RNA interference pathway (39, 40). By targeting mRNA transcripts post-translationally, they provoke either translational repression or degradation.

Analysis of miR expression revealed decreased expression of miR-29b, which has been shown to induce osteoblast differentiation. *In vitro* studies showed that miR-29b stimulates osteoblastogenesis by inhibiting protein expression of negative regulators of the cellular signal cascade transcription programmes, including for example TGFB3 (transforming growth factor beta) and DUSP2 (dual specificity protein phosphatase 2) (20). Mir-29b also stimulates osteogenic differentiation of unrestricted somatic stem cells from human cord

blood (41). We found not only downregulation of miR-29b but also upregulation of *TGFB3* and *DUSP2* mRNA.

Inhibition of miR-181a expression in subjects with insulin resistance in obesity also indicates bone defects. Recent studies presented overexpression of miR-181a in monocytes of lean persons. Moreover, downregulation of miR-181a expression was associated with a higher number of metabolic syndrome components (42, 43).

Transfection of sense miR-210-stimulated osteoblastic differentiation of murine stromal BM-MSC ST2 cells by positive regulation of the activin A receptor type-1B (AcvR1b), which inhibits TGF- β /activin signalling pathway (44). However, miR-210 expression was inhibited in whole blood from obese subjects with insulin resistance.

We also observed downregulation of miR-324-3p, which could suggest bone formation defects in subjects with insulin resistance. Recent studies presented that circulating miR-324-3p levels correlated with areal bone mineral density at the lumbar spine (18). Another study revealed positive association of miR-324-3p with bone microstructure and histomorphometry (19).

Summing up, results of our studies suggest defects in osteoblastogenesis in subjects with insulin resistance via mRNA downregulation for key signalling molecules in osteoblastogenesis, increased mRNA expression of osteoblastogenesis antagonists and inhibition of miRNA expression participating in regulation of osteoblasts differentiation. Defects in bone remodelling are reflected on protein levels by suppression of serum cOC decrease during OGTT in obese subjects with insulin resistance in comparison to individuals without insulin resistance. However, our study had some limitations. Firstly, the number of subjects included in the study was small and the number of both sexes was unequal. Secondly, mRNA and miRNA gene expression patterns due to financial limitations were analysed in a smaller group of subjects than biochemical parameters. However, the participants subjected to molecular tests reflected, in relation to the results of biochemical measurements, the whole two groups of subjects in the study (data not shown). One of the limitations of the study was also the fact that during OGTT we measured one marker of bone remodelling – osteocalcin, which is a non-collagenous protein produced by osteoblasts. This protein is considered a late marker of bone formation and increases at late stages of mineralisation. To reflect bone turnover other markers of bone formation (products of collagen synthesis (N-terminal propeptide of type I procollagen, PINP) and bone-specific alkaline phosphatase (BSAP)) as well as bone resorption (C-terminal telopeptide of type I collagen (CTX)) markers

should be measured. Last but not least, subjects included in the study did not have bone microstructure analysed. It could be interesting to confirm that inhibition of osteoblastogenesis gene and miR expression in whole blood of obese subjects with insulin resistance indeed reflect bone microstructure defects. However, we believe that our results could contribute to expand knowledge about circulating components of bone turnover and could be a basis for further studies evaluating bone remodelling defects in different disease states.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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