British Journal of Nutrition (2016), **116**, 1022–1032
doi:10.1017/S0007114516002993

Differences in metabolic biomarkers in the blood and gene expression profiles of peripheral blood mononuclear cells among normal weight, mildly obese and moderately obese subjects

Un Ju Jung[1], Yu Ri Seo[2], Ri Ryu[2] and Myung-Sook Choi[2,3]*

[1]*Department of Food Science and Nutrition, Pukyong National University, Busan 48513, Republic of Korea*
[2]*Department of Food Science and Nutrition, Kyungpook National University, Daegu 1370, Republic of Korea*
[3]*Center for Food and Nutritional Genomics Research, Kyungpook National University, Daegu 1370, Republic of Korea*

(Submitted 11 January 2016 – Final revision received 18 June 2016 – Accepted 14 July 2016 – First published online 9 August 2016)

Abstract

We compared metabolic biomarkers in the blood and peripheral blood mononuclear cell (PBMC) gene expression profiles among normal weight (BMI, 18·5–23 kg/m^2), mildly obese (BMI, 25–27·5 kg/m^2) and moderately obese Korean adult men (BMI, 27·5–30 kg/m^2). High leptin, lipids (except LDL- and HDL-cholesterol) and apoB levels and low adiponectin and HDL-cholesterol levels were present in the plasma of both mildly and moderately obese subjects. Circulating levels of inflammatory cytokines and markers of insulin resistance, oxidative stress and liver damage were altered in moderately obese subjects but not in mildly obese subjects. PBMC transcriptome data showed enrichment of pathways involved in energy metabolism, insulin resistance, bone metabolism, cancer, inflammation and fibrosis in both mildly and moderately obese subjects. Signalling pathways involved in oxidative phosphorylation, TAG synthesis, carbohydrate metabolism and insulin production; mammalian target of rapamycin, forkhead box O, ras-proximate-1, RAS and transforming growth factor-β signalling; as well as extracellular matrix–receptor interaction were enriched only in moderately obese subjects, indicating that changes in PBMC gene expression profiles, according to metabolic disturbances, were associated with the development and/or aggravation of obesity. In particular, fourteen and fifteen genes differentially expressed only in mildly obese subjects and in both mildly and moderately obese subjects, respectively, could be used as early or stable biomarkers for diagnosing and treating obesity-associated metabolic disturbance. We characterised BMI-associated metabolic and molecular biomarkers in the blood and provided clues about potential blood-based targets for preventing or treating obesity-related complications.

Key words: Transcriptomes: Peripheral blood mononuclear cells: Blood metabolic biomarkers: BMI: Obesity

Obesity is a growing health problem in both developed and developing countries[1]. Obesity increases the risk of numerous metabolic complications such as type 2 diabetes, dyslipidaemia, non-alcoholic fatty liver disease (NAFLD) and cancer[2]. BMI, a simple index of weight according to height, is commonly used to diagnose obesity in adults. Generally, people living in Western countries are considered obese when their BMI is 30 kg/m^2 or higher[2]. Some Asian countries use a lower BMI threshold for obesity (≥25 kg/m^2) because Asians are generally less obese but have higher body fat and insulin resistance than other ethnic groups with similar BMI[3,4]. A WHO expert consultation also suggested that Asian populations have different associations between BMI, percentage of body fat and health risks than European populations, and they identified intermediate public-health action points (BMI values of 23·0, 27·5, 32·5 and 37·5 kg/m^2) along the continuum of BMI and proposed methods by which countries could make decisions about the definitions of increased risk for their population[5]. Optimal BMI cut-off values for identifying metabolic risk are still controversial[6]. Therefore, further studies are needed to determine appropriate BMI cut-off values for Asians and to identify and prevent the development of obesity-related disorders.

Peripheral blood mononuclear cells (PBMC) can be easily obtained from blood samples. PBMC are mainly composed of lymphocytes and monocytes, cells that play key roles in the immune system. Bories *et al.*[7] reported that the expression of alternative macrophage differentiation markers with anti-inflammatory properties was decreased in PBMC from obese subjects compared with those from lean subjects, suggesting that PBMC in obesity are programmed and directly contribute to obesity-related inflammation. In addition, gene expression profiles of PBMC reflect lipid metabolism in obesity-associated organs such as the adipose tissue and the liver[8]. Therefore, numerous

Abbreviations: HOMA-IR, homoeostasis model assessment for insulin resistance; NAFLD, non-alcoholic fatty liver disease; PBMC, peripheral blood mononuclear cell.

* **Corresponding author:** M. S. Choi, fax +82 53 950 6229, email mschoi@knu.ac.kr

studies have attempted to identify transcriptional biomarkers in PBMC that may distinguish a disease state from a healthy state and predict pharmacodynamic effects[9,10].

This study aimed to establish metabolic and molecular differences among normal weight (BMI, 18·5–23 kg/m^2), mildly obese (BMI, 25–27·5 kg/m^2) and moderately obese (BMI, 27·5–30 kg/m^2) Korean adult men. We investigated the levels of lipids, apo and adipocytokines and markers of insulin resistance, oxidative stress and liver damage in plasma or erythrocytes. We also analysed gene expression profiles of PBMC using microarray analysis to understand molecular mechanisms underlying the development of obesity and its associated metabolic abnormalities.

Methods

Subjects

Volunteers aged 20–59 years were recruited from Daegu and its suburbs in the Republic of Korea. After an initial screening, thirty-seven men were included in the study. Of these, twenty-six were obese subjects having a BMI of 25–30 kg/m^2 and a normal medical history. Obesity was defined as a BMI of ≥25 kg/m^2 on the basis of the Asia-Pacific criteria set by the WHO[4]. Obese subjects were subdivided into two groups according to their BMI: mildly obese subjects (Obese A group, BMI from ≥25 to <27·5 kg/m^2; n 14)[1] and moderately obese subjects (Obese B group, BMI from ≥27·5 to <30 kg/m^2; n 12)[2]. A control group included eleven healthy age-matched subjects (BMI from ≥18·5 to <23 kg/m^2). Exclusion criteria included a history of cancer or cardiac, renal, hepatic or infectious disease; current treatment with insulin; current use of drugs for controlling blood glucose, blood lipids, body weight or high blood pressure; a history of gastrointestinal surgery; and consumption of functional foods or medications that may affect the results of this study. The study was performed in accordance with the Declaration of Helsinki, and all the subjects provided their written informed consent before participating. The study was approved by the Kyungpook National University's Human Research Committee (no. 2012-2), and all the experiments were performed in compliance with the relevant laws and institutional guidelines. The clinical trial registration number is ISRCTN16654407 (doi: 10.1186/ISRCTN16654407).

Analyses of plasma biomarkers and blood pressure

Blood samples were collected from the subjects after 12 h of fasting. Blood samples were collected in heparin-coated tubes and centrifuged at 1000 ***g*** and 4°C for 15 min to determine plasma levels of lipids, apo, glucose, insulin, C-peptide, glucagon, adipocytokines and aminotransferases. Levels of TAG, total cholesterol, HDL-cholesterol and glucose were determined using kits obtained from Asan Pharmaceutical Co., and levels of free fatty acids and phospholipids were determined using kits obtained from Wako Chemicals. LDL-cholesterol level was calculated using the Friedewald formula: total cholesterol − HDL-cholesterol − (TAG/5). The HDL-cholesterol:total cholesterol ratio (HTR) was calculated as (HDL-cholesterol/total cholesterol) × 100. The atherogenic index (AI) was calculated as (total cholesterol − HDL-cholesterol)/HDL-cholesterol. Levels of apoA1 and apoB were measured using enzymatic kits (*AlerChek* Inc.). Plasma levels of adipocytokines, insulin, C-peptide and glucagon were determined using a multiplex detection kit (Bio-Rad) and were analysed using the Luminex 200 LabMAP system (Luminex). Homoeostasis model assessment for insulin resistance (HOMA-IR) was calculated as (fasting glucose (mmol/l) × fasting insulin (μU/ml))/22·51. Plasma levels of aspartate aminotransferase (AST) and alanine transaminase (ALT) were determined using enzymatic kits (Asan Pharmaceutical Co.). Blood pressure was measured using an automatic blood pressure monitor (Omron).

Analyses of the activities of antioxidant enzymes

Activities of antioxidant enzymes were analysed as described previously[11]. Activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) in erythrocytes were determined as follows: heparin-treated fasting blood samples were centrifuged at 1000 ***g*** and 4°C for 15 min, and the plasma and buffy coat were discarded. The separated erythrocytes were washed three times with 0·9 % NaCl solution. The washed cells were lysed in an equal volume of distilled water and were mixed thoroughly. Hb concentration was estimated by analysing an aliquot of this haemolysate with a commercial assay kit (Asan Pharmaceutical Co.). The haemolysate was then diluted appropriately by adding distilled water to estimate the activities of CAT and GPX. Hb was removed by precipitation by adding 0·4 ml of an ethanol–chloroform (3:5, v/v) mixture to a 1-ml aliquot of the haemolysate cooled in ice. This mixture was stirred continuously for 15 min and diluted by adding 0·2 ml distilled water. After centrifuging for 10 min at 1600 ***g*** and 4°C, a pale yellow supernatant was separated from the protein precipitate and used to assay SOD activity.

SOD activity was spectrophotometrically measured by inhibiting pyrogallol autoxidation at 420 nm for 10 min; one unit of enzyme activity was determined as the amount of enzyme that inhibited the oxidation of pyrogallol by 50 %. CAT activity was measured spectrophotometrically by monitoring the disappearance of hydrogen peroxide at 240 nm for 5 min. GPX activity was determined spectrophotometrically by adding 100 μl of the diluted haemolysate to 2·8 ml of the reaction mixture containing 2·525 ml of 0·1 M-Tris-HCl (pH 7·2) buffer, 75 μl of 30 mM-glutathione, 100 μl of 6 mM-NADPH and 100 μl of glutathione reductase (0·24 units) and by incubating at 25°C for 5 min. The reaction was initiated by adding 100 μl of 30 mM-hydrogen peroxide, and the absorbance was measured at 340 nm for 5 min. Plasma paraoxonase activity was expressed as micromoles of *p*-nitrophenol produced per minute per millilitre of plasma.

Analysis of hydrogen peroxide and lipid peroxidation

The level of hydrogen peroxide in erythrocytes was analysed as previously described[11]. The ferrous oxidation-xylenol orange reagent was prepared by mixing 100 μM-xylenol

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orange, 250 μM-ammonium ferrous sulphate, 100 mM-sorbitol and 25 mM-H_2SO_4. Next, 50 μl of the test sample was added to 950 μl of the ferrous oxidation-xylenol orange reagent, vortexed and incubated at room temperature for at least 30 min to complete colour development. Absorbance was measured at 560 nm and compared with a linear standard having a concentration in the range of 0–5 μM. The level of thiobarbituric acid-reactive substance (TBARS), a marker of lipid peroxidation, was measured spectrophotometrically by using previously published methods[11].

Isolation of peripheral blood mononuclear cells and extraction of RNA

PBMC were isolated from heparin-treated blood samples by density-gradient sedimentation with Ficoll–Paque reagent (GE Healthcare) and used for total RNA extraction. Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The purity and integrity of the isolated RNA were evaluated using Agilent 2100 Bioanalyzer (Agilent Technologies).

Microarray analysis and real-time RT-PCR

For microarray analysis, we randomly selected eighteen subjects (four normal-weight subjects, seven mildly obese subjects and seven moderately obese subjects). Total RNA isolated from these subjects was amplified and purified using the Illumina TotalPrep RNA Amplification Kit (Ambion) to obtain biotinylated complementary RNA (cRNA). Next, 750 ng of biotinylated cRNA per sample was hybridised to Illumina HumanHT-12 v4 Expression BeadChips at 58°C for 16–18 h. Array signals were detected using Amersham Cy3–streptavidin (GE Healthcare). BeadChips were scanned using the Illumina BeadArray Reader, and raw data were extracted using Illumina BeadStudio software. Probe signal intensities were quantile normalised and log transformed. Limma was used to determine genes that were significantly differentially expressed based on a false-discovery rate < 5 %, a P value <0·05 and fold change >1·3. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (www.genome.jp/kegg) were considered for analysing gene functions. These microarray data were deposited in the Gene Expression Omnibus database (accession no. GSE69039).

To validate microarray data, six genes (*MMP9*, *IL-8*, *IL-1β*, IL-6, TNF-α and *HNRNPL*) were analysed independently by performing real-time RT-PCR with the same RNA samples as those hybridised with BeadChips. In all, 1 μg of total RNA was reverse transcribed using the QuantiTect® Reverse Transcription Kit (QIAGEN), and mRNA expression was quantified by performing real-time RT-PCR with the SYBR Green PCR Kit (QIAGEN) and the CFX96™ Real-Time PCR Detection System (Bio-Rad). Cycle thresholds (C_T) were determined on the basis of the SYBR Green emission intensity during the exponential phase. The C_T data were normalised using *GAPDH*, and relative gene expression was calculated using the $2^{-\Delta\Delta C_T}$ method.

Statistical analysis

All data are presented as mean values and standard deviations. Significant differences among the groups were determined using one-way ANOVA with SPSS (version 11.0; SPSS Inc.). Duncan's multiple range test was performed when differences among the groups were significant at $P < 0{\cdot}05$. Correlations between clinical parameters and the level of selected gene expression were assessed by Pearson's correlation test.

Results

General characteristics and plasma glucose-related markers of normal weight, mildly obese and moderately obese subjects

The general characteristics and plasma glucose-related markers are shown in Table 1. No significant differences were observed among the three groups with respect to age, systolic blood pressure, diastolic blood pressure, plasma glucose level and insulin:glucagon ratio. However, plasma insulin and glucagon levels tended to be higher in the Obese A group (BMI from ≥25 to <27·5 kg/m^2) than in normal weight subjects (BMI from ≥18·5 to <23 kg/m^2), and their plasma levels were significantly higher in the Obese B group (BMI from ≥27·5 to <30 kg/m^2) than in normal weight subjects. Moreover, the HOMA-IR of the Obese B group was significantly higher than that of normal weight subjects. The HOMA-IR of the Obese A group was intermediate between that of the normal weight and Obese B groups.

Plasma lipid and apo levels

Differences in plasma levels of lipids and apo are shown in Fig. 1. Plasma levels of free fatty acids, TAG, phospholipids, total cholesterol, non-HDL-cholesterol, apoB, apoB/apoA1 and AI were significantly higher in both Obese A and Obese B groups compared with the normal weight group. In particular, plasma levels of TAG, apoB and apoB/apoA1 in the Obese B group were significantly higher compared with the Obese A group. In contrast, levels of HDL-cholesterol and HTR were significantly lower in both obese groups than in the normal weight group. However, no significant differences were observed among the three groups with respect to plasma levels of LDL-cholesterol and apoA1.

Plasma levels of adipocytokines

Plasma levels of adipocytokines are shown in Fig. 2. Plasma levels of adiponectin in Obese A and Obese B groups were significantly lower compared with normal weight subjects. However, plasma levels of leptin in Obese A and Obese B groups were markedly higher than those in normal subjects. Moreover, the Obese B group showed significantly higher plasma levels of TNF-α and IL-6 than normal weight subjects; however, this was not observed in the Obese A group. Plasma levels of TNF-α and IL-6 in the Obese A group were approximately 5·29- and 1·25-fold higher, respectively, compared with normal weight subjects; however, the differences between Obese A and normal weight groups were not statistically significant. Plasma levels of resistin, monocyte chemoattractant

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Table 1. General characteristics and plasma glucose-related markers of obese and normal weight subjects (Mean values and standard deviations)

	Normal		Obese A		Obese B	
	Mean	SD	Mean	SD	Mean	SD
Number	11		14		12	
Age (years)	33·27	10·40	42·27	10·39	34·18	9·69
Body weight (kg)	63·79[a]	5·00	78·41[b]	2·34	88·07[c]	8·90
BMI (kg/m^2)	21·37[a]	1·36	25·79[b]	0·56	29·04[c]	2·51
Systolic blood pressure (mmHg)	126·67	2·08	120·33	5·13	141·20	15·90
Diastolic blood pressure (mmHg)	75·67	6·03	76·33	7·51	84·40	15·60
Plasma						
Glucose (mg/dl)	88·47	10·12	95·11	8·96	94·52	8·83
Insulin (ng/ml)	0·31[a]	0·13	0·46[a,b]	0·19	0·64[b]	0·33
C-peptide (ng/ml)	0·70[a]	0·19	1·12[a,b]	0·30	1·20[b]	0·44
Glucagon (ng/ml)	0·04[a]	0·03	0·05[a]	0·02	0·06[b]	0·01
Insulin/glucagon	9·05	4·01	9·57	3·77	9·95	7·98
HOMA-IR	1·67[a]	0·76	2·65[a,b]	1·18	3·83[b]	2·51

Normal, normal weight subjects; Obese A, mildly obese subjects; Obese B, moderately obese subjects; HOMA-IR, homoeostasis model assessment for insulin resistance.
[a,b,c] Mean values with unlike superscript letters were significantly different ($P<0·05$) (one-way ANOVA).

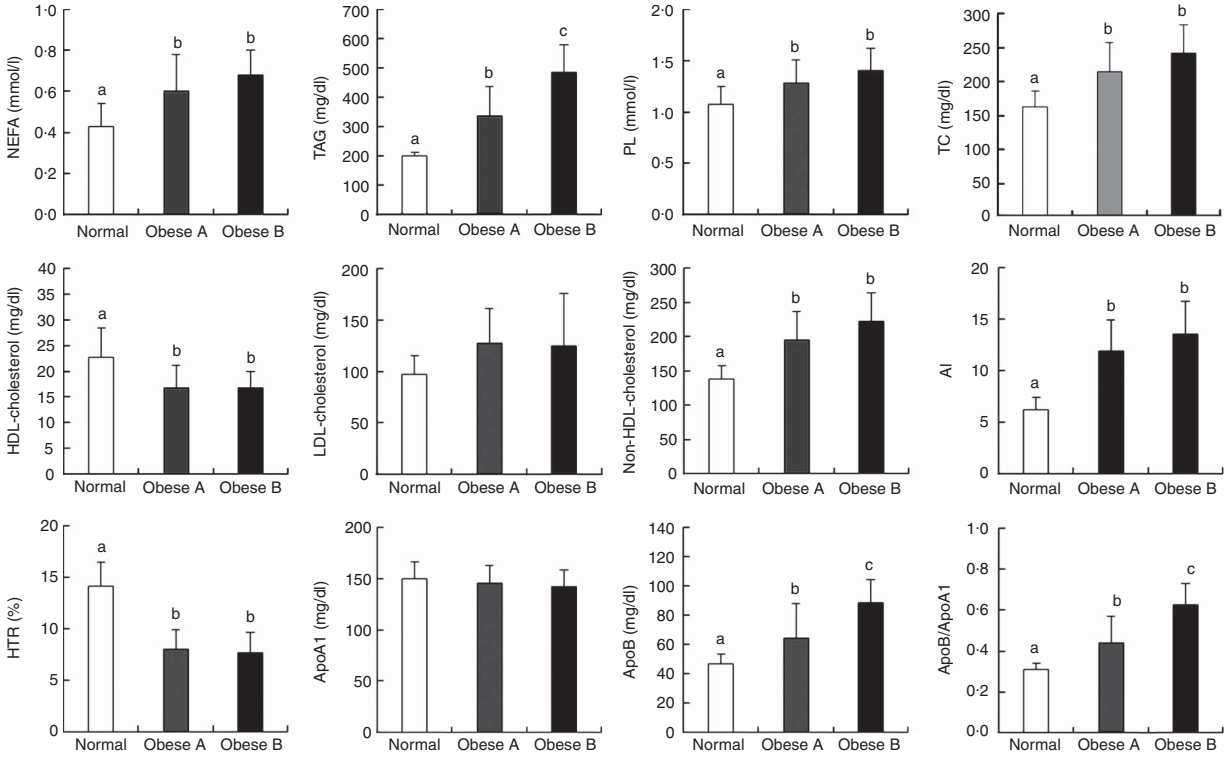

Fig. 1. Plasma levels of lipids and apo in obese and normal weight subjects. Subjects were categorised as normal weight (Normal), mildly obese (Obese A) and moderately obese (Obese B). Values are means and standard deviations represented by vertical bars. [a,b,c] Mean values with unlike letters were significantly different ($P<0·05$) (one-way ANOVA). AI, atherogenic index; HTR, HDL-cholesterol:total cholesterol ratio; PL, phospholipid; TC, total cholesterol.

protein-1, IL-1β and IL-8 were not significantly different among the three groups.

Activities of antioxidant enzymes and aminotransferases and levels of hydrogen peroxide and lipid peroxidation in plasma or erythrocytes

Similar to the plasma levels of HDL-cholesterol, the activity of plasma paraoxonase, a lipophilic antioxidant component of HDL-cholesterol, was significantly lower in Obese A and Obese B groups than in the normal weight group (Fig. 3(A)). Levels of oxidative stress indicators and activities of antioxidant enzymes in erythrocytes were not significantly different between Obese A and normal weight groups (Fig. 3(B) and (C)). However, the Obese B group showed markedly lower SOD and GPX activity and higher hydrogen peroxide and TBARS levels in erythrocytes compared with the normal weight group. However, erythrocyte CAT activity was not significantly

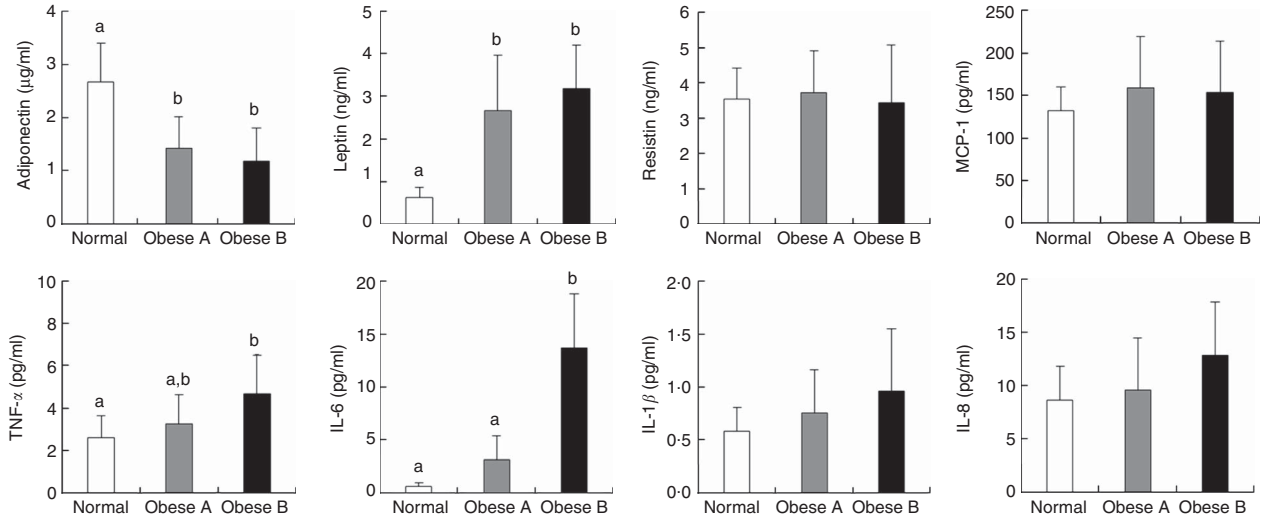

Fig. 2. Plasma levels of adipocytokines in obese and normal weight subjects. Subjects were categorised as normal weight (Normal), mildly obese (Obese A) and moderately obese (Obese B). Values are means and standard deviations represented by vertical bars. [a,b] Mean values with unlike letters were significantly different ($P < 0{\cdot}05$) (one-way ANOVA). MCP-1, monocyte chemoattractant protein-1.

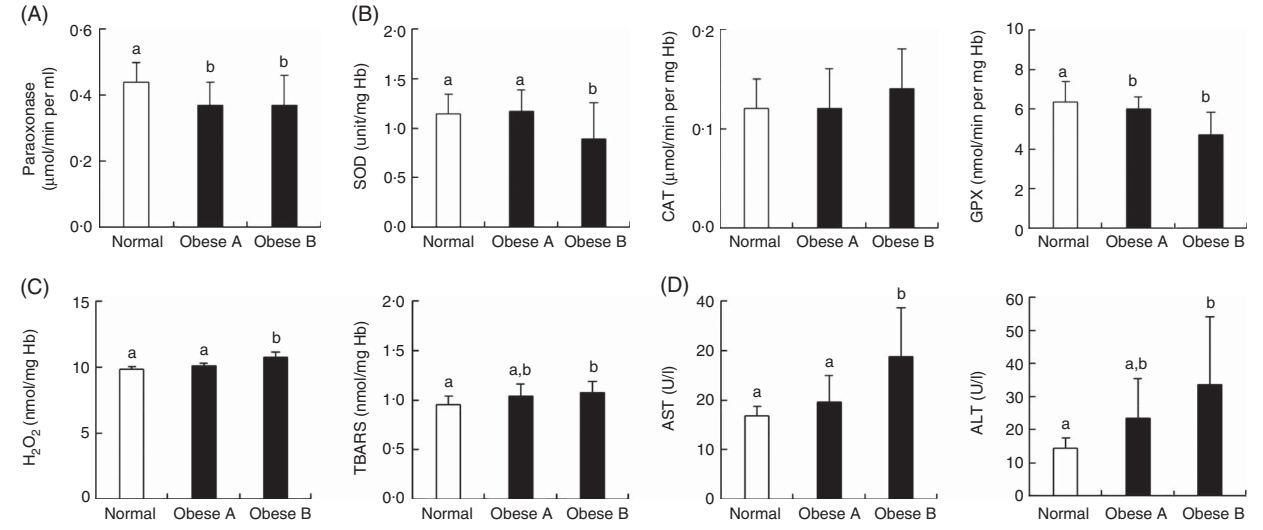

Fig. 3. Activities of antioxidant enzymes and levels of oxidative stress and liver function markers in plasma and/or erythrocytes of obese and normal weight subjects. Subjects were categorised as normal weight (Normal), mildly obese (Obese A) and moderately obese (Obese B). (A) Activities of antioxidant enzymes in plasma, (B) activities of antioxidant enzymes in erythrocytes, (C) levels of oxidative stress markers in erythrocytes and (D) activities of aminotransferases in plasma. Values are means and standard deviations represented by vertical bars. [a,b] Mean values with unlike letters were significantly different ($P < 0{\cdot}05$) (one-way ANOVA). ALT, alanine transaminase; AST, aspartate aminotransferase; CAT, catalase; GPX, glutathione peroxidase; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances.

different between the three groups. Parameters of liver function, such as plasma levels of AST and ALT, were also significantly higher in the Obese B group than in the normal weight group; however, these levels were not higher in the Obese A group (Fig. 3(D)).

Determination of gene expression profiles of peripheral blood mononuclear cells by microarray analysis

Microarray analysis identified genes that were differentially expressed in Obese A and Obese B groups compared with those in normal weight subjects. In all, eighty-four and 342 genes were up-regulated and fifty-five and 533 genes were down-regulated in Obese A and Obese B groups, respectively, compared with those in normal weight subjects (Fig. 4(a)). In all, seventy-four genes were common between the Obese A group and the Obese B group, of which thirty-six genes were up-regulated and thirty-eight genes were down-regulated. The ten most significantly up-regulated or down-regulated genes in Obese A and Obese B groups compared with those in normal weight subjects are shown in Fig. 4(b). In the Obese A group, the up-regulated gene with the highest fold change (1·87) was *CD83*, which is expressed in mature dendritic cells and activated B and T cells and plays an important role in

(a)

(b)

Obese A		Obese A	
Up-regulated genes	Fold change	Up-regulated genes	Fold change
CD83	1·87	*HBA1*	5·74
FCRLA	1·84	*ALAS2*	4·93
VPREB3	1·73	*ALPL*	4·45
CPT1B	1·72	*HBA2*	3·60
OSBPL10	1·71	*FCGR3B*	3·03
PIM3	1·69	*S100P*	2·97
ZNF101	1·67	*NAMPT*	2·90
TCP11L2	1·64	*MMP9*	2·64
CD19	1·62	*MME*	2·64
TACSTD2	1·59	*IL8*	2·60
Down-regulated genes	Fold change	Down-regulated genes	Fold change
SIGLEC14	–4·18	*KLRC2*	–4·07
CCL4L1	–1·97	*SIGLEC14*	–3·87
LOC645128	–1·83	*HNRNPL*	–3·08
LOC253039	–1·82	*LOC728715*	–2·86
GPR68	–1·66	*LOC441268*	–1·97
LOC100008588	–1·57	*CX3CR1*	–1·93
GIMAP8	–1·54	*CTAGE5*	–1·86
LPAR5	–1·52	*HSPA1A*	–1·85
HCP5	–1·52	*GPR68*	–1·83
CX3CR1	–1·51	*LGALS2*	–1·81

Fig. 4. Differential expression of genes in obese and normal weight subjects. Subjects were categorised as normal weight (Normal), mildly obese (Obese A) and moderately obese (Obese B). (a) Venn diagrams of genes that were significantly up-regulated (left) and down-regulated (right) in Obese A and Obese B groups compared with those in normal-weight subjects. (b) The ten most up-regulated or down-regulated genes in both Obese A and Obese B groups compared with those in normal weight subjects. Differentially expressed genes were determined using Limma in R/BioConductor based on a *P* value <0·05, false discovery rate <5 % and fold change >1·3.

modulating immune response[12], and the down-regulated gene with the lowest fold change (4·18) was *SIGLEC14*, which encodes a transmembrane protein of the Ig superfamily. However, the role of *SIGLEC14* in obesity is poorly understood. In the Obese B group, the up-regulated gene with the highest fold change (5·74) was *HBA1*, which encodes Hb, and the down-regulated gene with the lowest fold change (4·07) was *KLRC2*, which encodes a natural killer cell receptor and is down-regulated in immune response-mediated common inflammatory skin diseases such as atopic dermatitis[13]. The results of selected microarray analyses were confirmed by performing real-time RT-PCR (Fig. 5).

To further evaluate the functional differences in PBMC transcriptomes of Obese A and Obese B groups compared with those of normal weight subjects, we performed KEGG pathway analysis using genes that were differentially expressed only in the Obese A group, only in the Obese B group or in both Obese A and Obese B groups compared with those in normal weight subjects. Accordingly, differentially expressed genes only in Obese A group, only in Obese B group and in both Obese A and Obese B groups compared with those in normal weight subjects were used to perform the KEGG pathway analysis, respectively, and the results are indicated in Table 2. In the Obese A group, the differentially expressed

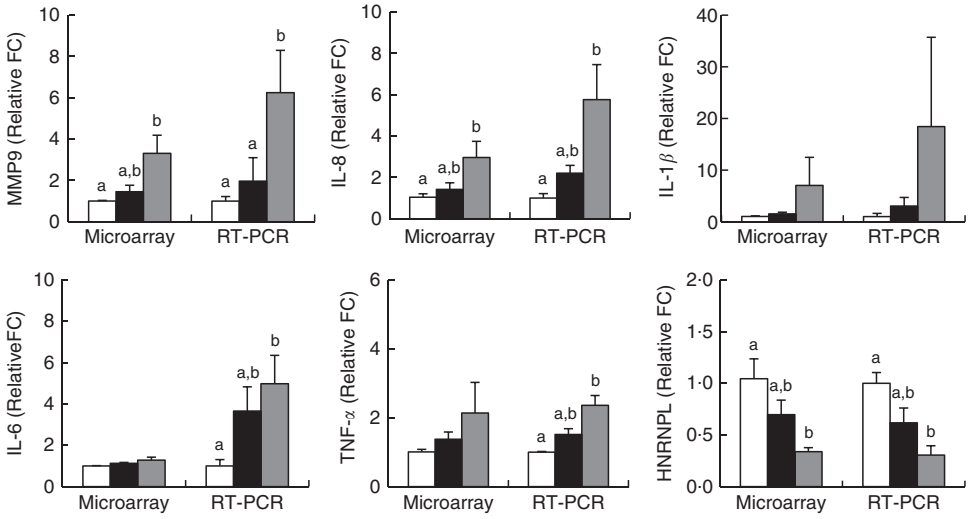

Fig. 5. Validation of microarray results by real-time RT-PCR. Subjects were categorised as normal weight (□, Normal), mildly obese (■, Obese A) and moderately obese (▩, Obese B). Values are means with their standard errors. [a,b] Mean values with unlike letters were significantly different ($P < 0{\cdot}05$) (one-way ANOVA). Differentially expressed genes were determined using Limma in R/BioConductor based on a P value <0·05, false discovery rate <5 % and fold change >1·3.

genes enriched pathways involved in immune responses, energy metabolism and cancer, such as cytokine–cytokine receptor interaction, NF-κB signalling, toll-like receptor (TLR) signalling, B cell receptor signalling, fatty acid metabolism, PPAR signalling, AMP-activated protein kinase signalling, mammalian target of rapamycin (mTOR) signalling, ras-proximate-1 signalling, calcium signalling, mitogen-activated protein kinase (MAPK) signalling, phosphoinositide-3-kinase (PI3K)–AKT signalling, RAS signalling and p53 signalling pathways. In particular, *CD19*, *CPT1B*, *CCL4L1* and *LPAR5* belonged to the top ten up- or down-regulated genes in the Obese A group compared with the normal weight group. In both Obese A and Obese B groups, the differentially expressed genes enriched pathways regulating NAFLD development, oestrogen signalling and osteoclast differentiation, along with pathways regulating immune responses and cancer, such as cytokine–cytokine receptor interaction, chemokine signalling, NF-κB signalling, TLR signalling, nucleotide-binding oligomerisation domain (NOD)-like receptor signalling, TNF signalling, T cell receptor signalling, B cell receptor signalling, transcriptional misregulation, Wnt signalling, MAPK signalling, p53 signalling, apoptosis and cAMP signalling; two of these genes (*CX3CR1* and *HSPA1A*) belonged to the top ten down-regulated genes in the Obese B group compared with the normal weight group. In addition, genes involved in extracellular matrix (ECM)-receptor interaction, transforming growth factor-β (TGF-β) signalling, TAG synthesis, oxidative phosphorylation, carbohydrate digestion and absorption, insulin signalling and forkhead box O (FOXO) signalling pathways were differentially expressed only in the Obese B group compared with the normal weight group, and *MMP9* and *FCGR3B* belonged to the top ten up-regulated genes in the Obese B group compared with the normal weight group. Microarray analysis of human PBMC transcriptomes showed that genes involved in pathways associated with immune responses; energy, glucose and bone metabolism; NAFLD; and cancer were differentially expressed during the early and/or late stages of obesity.

Next, from all the genes grouped in Table 2, we selected eight genes (*CD19*, *CPT1B*, *CCL4L1*, *LPAR5*, *CX3CR1*, *HSPA1A*, *MMP9* and *FCGR3B*) that belonged to the top ten up- or down-regulated genes in Obese A and Obese B groups compared with those in the normal weight group and analysed correlations between the selected eight genes and clinical parameters using Pearson's correlation test (Table 3). BMI was negatively correlated with *CX3CR1* and *HSPA1A*. Conversely, a positive correlation was observed between BMI and *MMP9* and *FCGR3B*. Among circulating lipids and apo, HDL-cholesterol level was negatively correlated with *CD19*, and levels of total cholesterol, non-HDL-cholesterol, LDL-cholesterol and apoB were positively correlated with *MMP9*, whereas LDL-cholesterol was negatively correlated with *CX3CR1*. In addition, *MMP9* expression showed positive correlations with levels of glucose-regulating hormones (insulin, C-peptide and glucagon) and the insulin resistance marker HOMA-IR, whereas *CX3CR1* expression was negatively correlated with levels of insulin, C-peptide and HOMA-IR. *MMP9* was also positively correlated with plasma leptin levels, whereas *LPAR5* was negatively correlated with IL-8 as well as leptin.

Discussion

Comparison of plasma lipids, apo, adipocytokines and markers of insulin resistance, oxidative stress and liver damage

To our knowledge, this is the first study to characterise metabolic biomarkers in the blood and transcriptome profiles of PBMC in Korean adults with mild or moderate obesity on the basis of the newly recommended BMI criteria for Asians[5]. Obese A and Obese B groups had abnormal levels of apoB, apoB/apoA1 and lipids, except LDL-cholesterol, which is

Table 2. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of genes that were significantly regulated in mildly and moderately obese Korean adult men*

KEGG pathway	Obese A alone†	Obese A and Obese B‡	Obese B alone§
Cytokine–cytokine receptor interaction	CCL28, LTB, CCL4L1, CCL3L1	CXCR5, FLT3LG, IL23A, CX3CR1, CCR2	CXCR1, EDAR, CXCR4, TNFRSF21, CCR7, CCR2, IL10, TNFSF10, CSF2RA, TNFSF14
Chemokine signalling pathway		NFKBIA, CXCR5	CXCR1, CXCR4, TSC22D3, GRB2, MAPK3, GNG10, ROCK2, PIK3CG, IKBKB
NF-κB signalling pathway	LTB, CCL4L1	TNFAIP3, NFKBIA, BIRC3	BCL10, RELB, TNFSF14, ERC1, IKBKB
TLR signalling pathway	CCL4L1, CCL3L1	NFKBIA	JUN, MAPK3, TLR5, PIK3CG, IKBKB
NOD-like receptor pathway		TNFAIP3, NFKBIA, BIRC3, NLRC4	RIPK2, MAPK3, IKBKB
TNF signalling pathway		TNFAIP3, NFKBIA, BIRC3	MMP9, JUN, BCL3, SOCS3, MAPK3, ITCH, BAG4, PIK3CG, CREB1, IKBKB, MLKL
T cell receptor signalling pathway		NFKBIA	JUN, BCL10, MAPK3, GRB2, CD28, ICOS, BCL10, MAPK3, PTPRC, PIK3CG, IL10, FYN, IKBKB
B cell receptor signalling pathway	CD19, CD79A	NFKBIA	JUN, BCL10, GRB2, MAPK3, DAPP1, PIK3CG, IKBKB
NAFLD		DDIT3	JUN, SOCS3, ITCH, PIK3CG, IKBKB
ECM-receptor interaction			CD44, CD47, TNC, ITGB1
TGF-β signalling pathway			MAPK3
Fatty acid metabolism	CPT1B, ELOVL6		PTPLAD2
PPAR signalling pathway	CPT1B		
AMPK signalling pathway	CPT1B,		PFKFB3, FOXO1, RAB10, PPP2R2B
mTOR signalling pathway	RRAGB		MAPK3, PIK3CG, IKBKB, RPS6KA3
TAG synthesis			
Oxidative phosphorylation			LHPP, PPA2
Carbohydrate digestion and absorption			MGAM, AMY2A, PIK3CG, AMY2B
Insulin signalling pathway			GRB2, SOCS3, MAPK3, FOXO1, FLOT1, PIK3CG, IKBKB
FOXO signalling pathway			GRB2, MAPK3, CDKN1B, FOXO1, TNFSF10, IL10, PIK3CG, IKBKB
RAP1 signalling pathway	LPAR5		MAPK3, LPAR1, PIK3CG, ITGB1
Oetrogen signalling pathway		HSPA1A	MMP9, JUN, GRB2, MAPK3, CREB1 PIK3CG, GABBR1
Osteoclast differentiation		NFKBIA	FCGR3B, JUN, GRB2, MAPK3, RELB, CAMK4, PIK3CG, CREB1, IKBKB, FYN
Calcium signalling pathway	CACNA1I		CAMK4, ATP2B4
Cancerpathway	GNG7, LPAR5	NFKBIA, BIRC3, FLT3LG, AXIN2	MMP9, CXCR4, JUN, TCF7, ABL1, GRB2, MAPK3, CDKN1B, GNG10, WNT7A, FOXO1, LPAR1, ROCK2, PIK3CG, CSF2RA, IKBKB, RASSF1, ITGB1
Transcriptional misregulation in cancer		BIRC3	MMP9, CCR7, CDKN1B, FOXO1, MEF2C, DUSP6, FLI1
Wnt signalling pathway		AXIN2	JUN, SIAH1, TCF7, WNT7A, ROCK2, DAAM1
MAPK signalling pathway	CACNA1I	DDIT3, HSPA1A, CACNA2D3,	JUN, GRB2, MAPK3, DUSP10, RELB, HSPA1A, MEF2C, IKBKB, RPS6KA3
PI3K–AKT signalling pathway	CD19, GNG7, LPAR5,		GRB2, MAPK3, CDKN1B, GNG10,
	PPP2R2B		CREB1, LPAR1, PIK3CG, TNC, IKBKB, ITGB1
RAS signalling pathway	GNG7		ABL1, GRB2, MAPK3, GNG10, PIK3CG, RASSF1, IKBKB
p53 signalling pathway	RPRM	APAF1	SIAH1, SESN3, ZMAT3
Apoptosis		NFKBIA, BIRC3, APAF1	TNFSF10, PIK3CG, IKBKB
cAMP signalling pathway		PDE4B, NFKBIA	JUN, MAPK3, CAMK4, CREB1, ROCK2, LPAR1, PIK3CG, ATP2B4, PDE4C

TLR, toll-like receptor; NOD, nucleotide-binding oligomerisation domain; NAFLD, non-alcoholic fatty liver disease; ECM, extracellular matrix; TGF-β, transforming growth factor-β; AMPK, AMP-activated protein kinase; mTOR, mammalian target of rapamycin; FOXO, forkhead box O; RAP1, ras-proximate-1; MAPK, mitogen-activated protein kinases; PI3K–AKT, phosphoinositide-3-kinase-AKT; cAMP, 3′-5′-cyclic adenosine monophosphate.

* Analyses were performed using KEGG pathway analysis (www.genome.jp/kegg) with genes that were differentially expressed only in the mildly obese (Obese A) group, only in the moderately obese (Obese B) group or in both Obese A and Obese B groups compared with those in normal weight subjects. Genes in red font are those up-regulated in obese subjects compared with those in normal weight subjects; genes in green font are those down-regulated in obese subjects compared with those in normal weight subjects; the underlined genes belong to the top ten up- or down-regulated genes.

† Genes differentially expressed only in the Obese A group compared with those in normal weight subjects.

‡ Genes differentially expressed in both Obese A and Obese B groups compared with those in normal weight subjects.

§ Genes differentially expressed only in the Obese B group compared with those in normal weight subjects.

consistent with previous findings showing that apoB, apoB/apoA1 and non-HDL-cholesterol levels may be more clinically useful than LDL-cholesterol levels for predicting the risk of CVD in obese subjects[14]. In particular, plasma levels of apoB, apoB/apoA1 and TAG were significantly higher in the Obese B group than in the Obese A group, indicating that these blood markers may be more sensitive to the severity of obesity. Increased plasma levels of leptin and decreased plasma levels of adiponectin were observed in both Obese A and Obese B groups. As leptin (positively) and adiponectin (negatively) are correlated with body fat, BMI and dyslipidaemia[2], they could be suitable sensitive biomarkers of body fat accumulation and early risk of obesity-associated dyslipidaemia in obese subjects. Unlike plasma levels of lipids, apoB and adipokines, plasma levels of inflammatory cytokines and markers of insulin resistance, oxidative stress and liver damage were significantly increased in the Obese B group but not in the Obese A group. Although both Obese A and Obese B groups maintained normal fasting blood glucose levels because of increased plasma glucagon and insulin levels, the HOMA-IR of the Obese B group was markedly higher than that of the normal weight group, suggesting that changes in HOMA-IR were more sensitive in predicting the severity of obesity than levels of fasting blood glucose. Thus, a part of our findings suggests that dysregulation of circulating levels of lipids, apoB, leptin and adiponectin may occur before the changes in the circulating levels of markers of inflammation, insulin resistance, oxidative stress and liver function, and thus may be more efficient in predicting the early risk of obesity and associated metabolic diseases. Similarly, circulating levels of adiponectin are suggested to precede the decrease in insulin sensitivity in humans[15].

Table 3. Correlation of clinical parameters and selected genes

	CD19	*CPT1B*	*CCL4L1*	*LPAR5*	*CX3CR1*	*HSPA1A*	*MMP9*	*FCGR3B*
BMI	0·090	0·155	0·060	−0·354	−0·630**	−0·668**	0·639**	0·570*
Total cholesterol	0·038	−0·211	−0·451	0·074	−0·373	−0·254	0·684**	0·343
HDL-cholesterol	−0·544*	−0·318	0·352	0·103	−0·092	0·295	0·142	0·154
Non-HDL-cholesterol	0·157	−0·165	0·423	0·059	−0·359	−0·295	0·661**	0·319
LDL-cholesterol	0·156	−0·212	0·179	0·012	−0·489*	−0·062	0·622**	0·294
apoB	0·170	−0·232	0·221	0·039	−0·361	−0·053	0·585*	0·323
Insulin	0·290	−0·102	0·308	−0·115	−0·521*	−0·135	0·625**	0·325
C-peptide	0·158	0·078	0·137	−0·217	−0·579*	−0·206	0·525*	0·285
Glucagon	0·021	0·122	0·062	−0·337	−0·428	−0·041	0·516*	0·544*
HOMA-IR	0·323	−0·178	0·391	−0·055	−0·481*	−0·158	0·646*	0·331
Leptin	0·067	0·389	−0·182	−0·541*	−0·323	0·004	0·519*	0·400
IL-8	0·072	0·370	−0·244	−0·559*	−0·097	0·089	0·102	0·110
Paraoxonase	−0·472*	−0·314	0·327	0·003	−0·329	−0·136	0·160	0·183

HOMA-IR, homoeostasis model assessment for insulin resistance.
**, *Significant at $P = 0·01$, $P = 0·05$, respectively.

Comparison of inflammatory and fibrotic gene expressions in peripheral blood mononuclear cells

Importantly, gene expression profiles of PBMC reflected metabolic differences associated with obesity status. As expected, multiple genes involved in inflammatory pathways were differently expressed in PBMC from Obese A and/or Obese B subjects. Up-regulation of *LTB*, *CCL28*, *CD19* and *CD79A* and down-regulation of *CCL4L1* and *CCL3L1* were observed only in the Obese A group, suggesting that these genes may be involved in the early stage of obesity-related inflammation. In particular, *CD19* was negatively correlated with plasma HDL-cholesterol as well as paraoxonase, a lipophilic antioxidant component of HDL-cholesterol, suggesting a possible link between this gene and HDL-cholesterol dysregulation. Further, of the three genes *CX3CR1*, *CCR2* and *NLRC4*, which were down-regulated in both Obese A and Obese B groups, loss of *CX3CR1* (encoding a fractalkine receptor) induces hyperglycaemia with reduced insulin secretion[16]. In contrast, fractalkine improves glucose tolerance and insulin secretion *in vivo*[16], and polymorphisms in *CX3CR1* (T280M and V249I) are associated with type 2 diabetes[17]. Interestingly, in the present study, a negative correlation was observed between *CX3CR1* expression and levels of plasma glucose-regulating hormones (insulin, C-peptide and glucagon) and HOMA-IR, thus supporting the functional role of this gene in the metabolic syndrome. More inflammation-related genes were differentially expressed in the Obese B group than in the Obese A group. In particular, expression of anti-inflammatory *IL10* was down-regulated only in the Obese B group – a finding similar to that observed in a previous study[7]. In addition, expressions of pro-inflammatory cytokines such as *IL6* and *TNFα* in PBMC were significantly up-regulated only in the Obese B group, which was consistent with their circulating levels.

Along with inflammation, increased fibrosis of the liver and adipose tissue is a hallmark of obesity, and liver fibrosis is a component of NAFLD[18]. Interestingly, NAFLD-related genes were differentially expressed in PBMC of obese subjects. Of these, an increased expression of endoplasmic reticulum stress-related *DDIT3*, a gene commonly up-regulated in both Obese A and Obese B groups, is observed in PBMC of type 1 diabetic subjects[19], whereas a deficiency of *DDIT3* reduces oxidative damage and promotes cell survival in type 2 diabetic mice[20]. Moreover, besides the profibrotic mediator MMP9, other genes involved in the fibrotic process such as ECM–receptor interaction and TGF-*β* signalling were differentially expressed only in the Obese B group. In particular, expression of *MMP9*, a gene up-regulated only in the Obese B group, showed positive correlations with levels of plasma lipids (total cholesterol, non-HDL-cholesterol and LDL-cholesterol), apo B, glucose-regulating hormones (insulin, C-peptide and glucagon), insulin resistance marker (HOMA-IR) and leptin as well as BMI, suggesting a link between obesity and related metabolic disturbances such as dysregulation of lipid and glucose metabolism. Recently, Mazzotti *et al.*[21] also reported an association of *MMP9* polymorphisms with higher LDL-cholesterol levels. Among other TGF-*β* signalling-related genes down-regulated only in the Obese B group, loss of *CD44* or *CD47* induced adiposity or impaired glucose tolerance in mice fed a high-fat diet[22,23]. In addition, *CD47* expression on the erythrocyte surface was lower in obese patients than in the control group[24].

Comparison of expression of genes involved in energy metabolism, insulin resistance and bone metabolism in peripheral blood mononuclear cells

Numerous genes involved in energy metabolism, except *CPT1B*, were also differentially expressed in PBMC of the Obese B group. In particular, the Obese B group showed up-regulation of *FOXO1*, which regulates energy and nutrient homoeostasis through energy storage in white adipose tissue[25]. In mice, *FOXO1* haploinsufficiency protected against high-fat diet-induced insulin resistance[26]. Moreover, inhibition of *PPA2*, which was down-regulated only in the Obese B group, was reported to exacerbate insulin resistance[27]. In addition, various genes involved in carbohydrate digestion and absorption and insulin resistance were differentially expressed only in the Obese B group, consistent with findings regarding the circulating levels of glucose-related markers. Up-regulation of *CPT1B*, which encodes a *β*-oxidation enzyme, only in the Obese A group may be an essential metabolic response for maintaining lipid balance in the early stages of obesity[28].

Accordingly, *CPT1B* may be an early complementary marker that protects against obesity-associated lipid dysregulation, and more genes and pathways involved in energy and glucose metabolism may reflect dysregulation of energy homoeostasis and insulin resistance in later stages of obesity.

Oestrogen signalling in men, as in women, prevents adiposity and insulin resistance[29,30]. Interestingly, both Obese A and Obese B groups showed down-regulation of oestrogen signalling-related *HSPA1A*, which is also down-regulated in the muscles of patients with type 2 diabetes and protects against obesity-induced insulin resistance[31,32]. Loss of *GABBR1* (involved in oestrogen signalling) or *ATP2B4* (involved in calcium signalling), which was down-regulated only in the Obese B group, increases insulin resistance and hepatic steatosis and osteoclastogenesis with a concomitant reduction in bone volume in mice[33,34]. Therefore, these PBMC genes may be associated with obesity-related metabolic disturbances and bone metabolism dysregulation.

Comparison of expression of genes involved in cancer in peripheral blood mononuclear cells

Obesity-related enhancement in growth signalling, inflammation and angiogenesis can contribute to cancer progression[35]. The Wnt, MAPK, PI3K-AKT, p53, mTOR and RAS pathways are involved in several aspects of tumourigenesis, such as cell proliferation, survival, migration, invasion and angiogenesis[36]. In particular, p53 controls apoptosis, and p53-dependent apoptosis suppresses tumourigenesis[37]. Of the genes involved in these pathways, up-regulation of *CACNA1I*, *CD19*, *GNG7* and *RPRM* and down-regulation of *LPAR5* and *PPP2R2B* were observed only in the Obese A group. *CD19* is over-expressed in various cancers, whereas *LPAR5* protects against tumour formation[38,39]. In this case, the up-regulation of oncogenes *AXIN2, FLT3LG*, *NFKBIA*, *BIRC3*, *DDIT3*, and *PDE4B* and down-regulation of suggested tumour-suppressor genes *CACNA2D3*, *HSPA1A* and *APAF1* occurred continuously in both Obese A and Obese B groups[40–48]. Moreover, more cancer-related genes were expressed in PBMC from the Obese B group than in PBMC from the Obese A group, indicating that after cancer initiation in the early stage of obesity, this ongoing process drives cancer towards more invasive stages. Thus, the PBMC genes identified in our study could be used as potential targets for preventing, treating and diagnosing obesity-related cancers.

In conclusion, we examined, for the first time, the changes in metabolic features and gene expression profiles of PBMC according to BMI in adult Korean men. The Obese B group showed overall characteristics of the metabolic syndrome, including dyslipidaemia, adipocytokine dysregulation, insulin resistance, increased oxidative stress and NAFLD markers, whereas the Obese A group only showed dyslipidaemia and abnormalities in the levels of leptin and adiponectin. Moreover, genes involved in energy metabolism, insulin resistance, bone metabolism, cancer, inflammation and fibrosis were differentially expressed in the PBMC of obese subjects. Of these, fourteen genes differentially expressed only in the Obese A group may be used as potential targets for the early diagnosis and treatment of obesity-related metabolic disturbances. We also identified fifteen genes associated with inflammation, NAFLD and cancer that were common between the Obese A group and the Obese B group, implying that expression and stability of these genes may be associated with obesity-related metabolic disorders. Thus, our findings could be potentially useful for diagnosing and managing obesity. However, further larger-scale studies are required to completely elucidate the role of these genes in the pathogenesis of obesity.

Acknowledgements

This study was supported by the SRC programme (grant number 2015R1A5A6001906) and the Fundamental Technology Program (grant number 2012M3A9C4048818) of the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology.

This study received no specific grant from any funding agency, commercial or not-for-profit sectors. In addition, all the funders had no role in the design and analysis of the study or in the writing of this article.

U. J. J. analysed the data and wrote the manuscript. Y. R. S. and R. R. performed the clinical study and were involved in data interpretation. M.-S.C. designed the study and edited the manuscript. All the authors read and approved the final version of the manuscript.

The authors declare that there are no conflicts of interest.

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