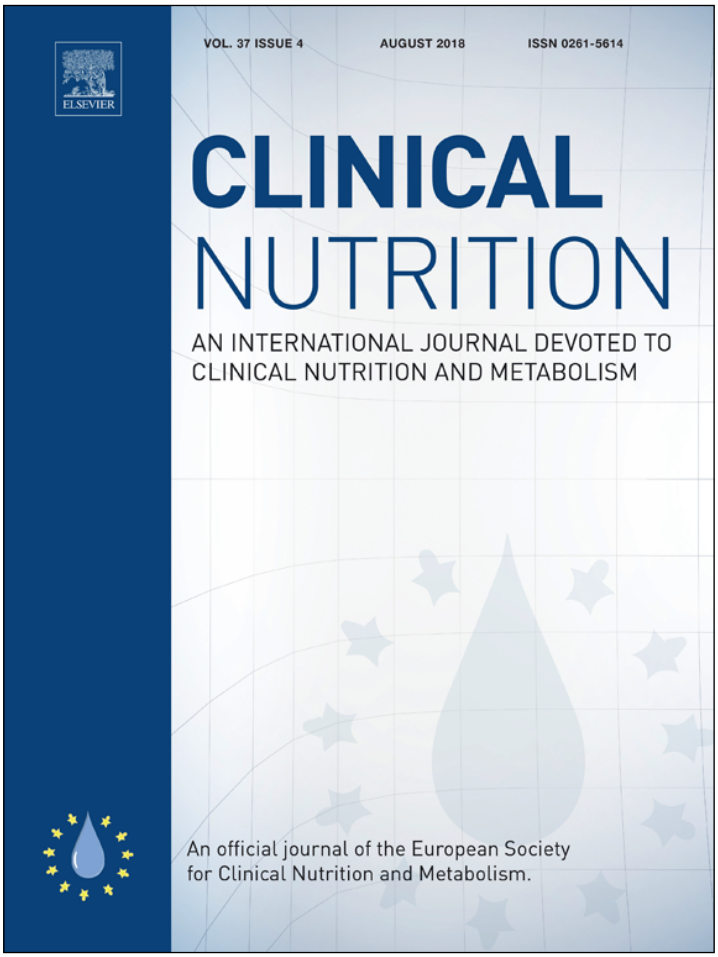

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Original article

Gene expression analysis identify a metabolic and cell function alterations as a hallmark of obesity without metabolic syndrome in peripheral blood, a pilot study

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SUMMARY

Background: Understanding molecular basis involved in overweight is an important first step in developing therapeutic pathways against excess in body weight gain.
Objective: The purpose of our pilot study was to evaluate the gene expression profiles in the peripheral blood of obese patients without other metabolic complications.
Design: A sample of 17 obese patients without metabolic syndrome and 15 non obese control subjects was evaluated in a prospective way. Following 'One-Color Microarray-Based Gene Expression Analysis' protocol Version 5.7 (Agilent p/n 4140-90040), cRNA was hybridized with Whole Human Genome Oligo Microarray Kit (Agilent p/n G2519F-014850) containing 41,000+ unique human genes and transcripts.
Results: The average age of the study group was 43.6 ± 19.7 years with a sex distribution of 64.7% females and 35.3% males. No statistical differences were detected with healthy controls 41.9 ± 12.3 years with a sex distribution of 70% females and 30% males. Obese patients showed 1436 genes that were differentially expressed compared to control group. Ingenuity Pathway Analysis showed that these genes participated in 13 different categories related to metabolism and cellular functions. In the gene set of cellular function, the most important genes were C-terminal region of Nel-like molecule 1 protein (NELL1) and Pigment epithelium-derived factor (SPEDF), both genes were over-expressed. In the gene set of metabolism, insulin growth factor type 1 (IGF1), ApoA5 (apolipoprotein subtype 5), Foxo4 (Forkhead transcription factor 4), ADIPOR1 (receptor of adiponectin type 1) and AQP7 (aquaporin channel proteins7) were over expressed. Moreover, PIKFYVE (PtdIns(3) P 5-kinase), and ROCK-2 (rho-kinase II) were under expressed.
Conclusion: We showed that PBMCs from obese subjects presented significant changes in gene expression, exhibiting 1436 differentially expressed genes compared to PBMCs from non-obese subjects. Furthermore, our data showed a number of genes involved in relevant processes implicated in metabolism, with genes presenting high fold-change values (up-regulation and down regulation) associated with lipid, carbohydrate and protein metabolism.

1. Introduction

Understanding molecular basis involved in obesity is an important first step in developing treatments against excess in body weight gain [1,2]. Based on this, the study of gene expression at the level of mRNA is being carried out by transcriptomics, mainly using arrays [3]. In this context, most gene expression studies in human obesity are often focused on adipocytes, mainly on biopsied subcutaneous adipose tissue [4] and analysis of visceral adipose tissue is basically restricted to morbid patients during surgery, [5]. However, these studies in obese patients have remained clearly

List of abbreviations: ADIPOR1, receptor of adiponectin type 1; ApoA5, apolipoprotein subtype 5; AQP7, aquaporin channel proteins7; BMI, body mass index; IGF1, insulin growth factor type 1; IPA, Ingenuity Pathway Analysis; Foxo4, Forkhead transcription factor 4; MS, Metabolic syndrome; NELL1, Nel-like molecule 1 protein; PBMC, peripheral blood mononuclear cells; PIKFYVE, PtdIns(3) P 5-kinase; ROCK-2, rho-kinase II; SPEDF, Pigment epithelium-derived factor.

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insufficient to date because it is difficult to obtain biopsies from every volunteer involved in a nutritional study due to the invasive sample collection procedure [6].

Molecular pathways underlying several human diseases have been investigated by gene expression analysis of peripheral blood mononuclear cells (PBMC) as surrogates for predicting potential effects in tissues that are difficult to access [7]. This tissue (mononuclear cells) has been proposed as a useful tool to better understand multiple pathways; such as oncology [8] and cardiovascular research [9]. The suitability of PBMCs for genomics studies in the obesity topic area is in part related to their active metabolism and accessibility by a simple venipuncture [10]. Interestingly, a remarkable concordance (80–90%) of gene expression profiles between PBMC and different tissues has been demonstrated [10]. Therefore, transcriptome analysis of peripheral blood is a promising approach for determining disease and treatment outcome. One study in the literature [11] has evaluated the peripheral blood gene expression in patients with metabolic syndrome or diabetes mellitus type 2, but as far as we know the profile has not been evaluated in patients with obesity without other metabolic complications. The aim of our pilot study was to investigate the peripheral blood gene expression profiles in obese patients without other metabolic complications.

2. Material and methods

2.1. Subjects and procedures

This study was realized according to the guidelines laid down in the Declaration of Helsinki and it was approved by the HURH ethics committee. A sample of 17 obese patients without metabolic syndrome and 15 healthy control subjects was enrolled in a prospective way. These patients were enrolled in a Nutrition Clinic Unit. All participants provided informed consent to a protocol approved by the local ethical review boards. In obese subjects, inclusion criteria were body mass index $\geq$30 and exclusion criteria included history of cardiovascular disease or stroke, fasting plasma glucose $\geq$110 mg/dl or diabetes mellitus, or presence of metabolic syndrome (MS) diagnosed by the definitions of the ATPIII was considered [12]. The cutoff points for the criteria used to define MS are 3 or more of the following; central obesity (waist circumference >88 cm in women and >102 cm in men), hypertriglyceridemia (triglycerides >150 mg/dl or specific treatment), high density lipoprotein cholesterol <50 mg/dl, hypertension (systolic blood pressure >130 mmHg or diastolic Blood Pressure >85 mmHg or specific treatment) or fasting plasma glucose >100 mg/dl or drug treatment for elevated blood glucose. The healthy control group was subjects who had never been diagnosed with a chronic illness, without pharmacological treatments and body mass index was under 25 kg/m^2.

Weight, height, body mass index (BMI) and blood pressure measures were realized within the start of the trail. These measures were realized at same time of the day (morning).

2.2. Anthropometric measurements and blood pressure

Blood pressure was measured twice after a 10 min rest with a random zero mercury sphygmomanometer, and averaged (Omron, LA, CA). Body weight was measured to an accuracy of 0.1 kg and body mass index computed as body weight/(height^2). Waist circumference was measured, too.

2.3. Biochemical determinations

Blood serum was obtained after 10 h of fasting state. Serum total cholesterol and triglyceride concentrations were determined by enzymatic colorimetric assay (Technicon Instruments, Ltd., New York, N.Y., USA), while high density lipoprotein cholesterol was determined enzymatically in the supernatant after precipitation of other lipoproteins with dextran sulfate–magnesium. Low density lipoprotein cholesterol was calculated using Friedewald formula. Plasma glucose levels were determined by using an automated glucose oxidase method (Glucose analyser 2, Beckman Instruments, Fullerton, California). Insulin was measured by RIA (RIA Diagnostic Corporation, Los Angeles, CA) with a sensitivity of 0.5 mUI/L (normal range 0.5–30 mUI/L) and the homeostasis model assessment for insulin resistance (HOMA-IR) was calculated using this formula (fasting insulin $\times$ fasting glucose concentrations/22.5).

2.4. Microarrays assays

After 10 h in fasting state, a sample of 2.5 ml of peripheral whole blood was collected and drawn directly into PaxGene venous blood vacuum collection tubes (Becton Dickinson, USA). Total RNA was extracted from blood samples using the PAXgene Blood RNA System (PreAnalytix, Hombrechtikon, Switzerland). RNA was quantified and quality confirmed by RNA 6000 Nano Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) assay. Up to 1750 ng of each RNA sample was concentrated with the RNeasy MinElute Cleanup kit (QIAGEN, Hilden, Germany). RNA was eluted with 10 ml of RNase-free H_2O. 100 ng of purified total RNA were used to produce Cyanine 3-CTP-labeled cRNA using the Quick Amp Labeling kit (Agilent p/n 5190-0442) according to the manufacturer's instructions. Following 'One-Color Microarray-Based Gene Expression Analysis' protocol Version 5.7 (Agilent p/n 4140-90040), 3 μg of labeled cRNA was hybridized with Whole Human Genome Oligo Microarray Kit (Agilent p/n G2519F-014850) containing 41,000+ unique human genes and transcripts. Arrays were scanned in an Agilent Microarray Scanner (Agilent G2565BA) according to the manufacturer's protocol and data extracted using Agilent Feature Extraction Software 9.5.3 following the Agilent protocol GE1-v5_95_Feb07 and the QC Metric Set GE1_QCMT_Jan08.

Resulting microarray datasets were uploaded at the Array Express microarray data repository (E-MTAB-3017). Changes were evaluated in microarray gene expression for two representative genes of our analysis by qPCR, using B2M as reference (housekeeping) gene, following the manufacturer's protocol (Real-time Ready, Roche Applied Science, Germany).

2.5. Microarray data analysis

Data analysis was realized in Research Unit, Hospital Clínico Universitario-IECSCYL, Valladolid, Spain by using GeneSpring GX 11.0 software. The original data was cleansing and normalized as previously described [13]. The baseline transformation of the data was realized using the median of all samples.

Before statistical analyses all microarrays were subjected to quality and filtering criteria. Quality of the microarray data was assessed on principal component analysis (PCA) plots. All the 32 arrays passed these criteria and were included in the analyses. Mann–Whitney test was used to identify genes differentially expressed between different groups at the level of significance $p < 0.05$ with Benjamini–Hochberg multiple testing corrections and considering a Fold change = +2 as threshold. Functional analyses were realized using Ingenuity Pathway Analysis 8.5 (IPA) (Ingenuity Systems, Redwood City, CA) software. For IPA analyses, only significantly differentially datasets obtained in the GeneSpring analyses were analyzed. GeneSpring GX 11.0 was used also for performing gene hierarchical clustering.

3. Results

In the present study, we evaluated the gene expression profile displayed by PBMC's from obese patients without metabolic disorders with that healthy subject without obesity. The average age of the study group was 43.6 ± 19.7 years with a sex distribution of 64.7% females and 35.3% males. These dates' didn't show statistical differences with non-obese subjects 41.9 ± 12.3 years with a sex distribution of 70% females and 30% males. Epidemiological characteristics have been showed in Table 1.

The total number of leukocytes and lymphocytes was similar in both groups: (obese: 2784 ± 1235/uL vs control 2683 ± 935/uL), (obese: 976 ± 299/uL vs control 901 ± 233/uL), respectively. The volcano plot analysis showed differences in mRNA expression levels between obese and non-obese groups considering FDR corrected p-value cutoff <0.05 and fold-change cutoff = 2 (up- or down-regulated). We identified 1436 differentially expressed genes: 1248 genes with relative higher expression levels and 168 with lower expression levels (Fig. 1). The majority of differentially expressed genes in PBMCs from obese patients showed expression levels lower than 2.5-fold change (up or down) when compared to non-obese subjects, and a few genes showed FC ≥ 2.5. Changes in gene expression observed by microarrays were validated for two representative genes of our analysis. Gene expression levels of PtdIns(3) P 5-kinase and rho-kinase II measured by quantitative RT-PCR were compared using Mann–Whitney test (significance level was fixed at 0.05) (Supplementary data 1).

Additionally, when all samples were analyzed together, the hierarchical clustering analysis indicate that 15/15 control subjects cluster, on one branch with four obese patients (Fig. 2) and these obese patients had lower body mass index (33, 32.5, 31 and 32 kg/m^2) than the remainder obese patients. The second branch features just all the remainder obese patients.

Taking to account that hierarchical clustering reported difference in gene expression profiles of each group and potential overlap amongst the signatures of the metabolic states, we further studied the relationships of gene expression within and amongst the 2 states in the context of gene sets. A gene set is defined as a group of genes with a common aim, derived from the Gene Ontology project [14]. These canonical pathways have been evaluated with the molecular and cell function algorithm of GeneSpring and a total of 13 categories has been detected (Table 2) and these groups have been joined in two: “cellular function” and “metabolism”. Cluster analysis showed 88 genes in lipid metabolism, 66 genes in carbohydrate metabolism, 7 genes in protein metabolism and 25 genes in vitamin–mineral metabolism (Fig. 3).

Table 1
Anthropometric and biochemical parameters in obese patients vs healthy controls.

Characteristics	Obese patients (n = 17)	Healthy control (n = 15)
BMI	37.2 ± 4.4	21.5 ± 3.9*
Sex (Male/female)	6/11	5/15
Weight (kg)	100.6 ± 20.8	68.5 ± 19.2*
WC (cm)	107.1+10.3	7.2 + 10.6*
Systolic BP (mmHg)	122.2+9.7	121.6 + 11.1
Diastolic BP (mmHg)	83.6+6.3	85.1 + 7.0
Glucose (mg/dl)	97.1 ± 7.9	91.2 ± 21.5
Total ch. (mg/dl)	203.7 ± 43.6	202.3 ± 31.9
LDL-ch. (mg/dl)	120.7 ± 35.8	119.7 ± 31.1
HDL-ch. (mg/dl)	53.1 ± 12.9	51.4 ± 20.3
TG (mg/dl)	124.1 ± 34.7	122.1 ± 31.2
Insulin (UI/L)	8.1 ± 4.9	6.4 ± 3.3
HOMA-IR (units)	2.9 ± 1.9	2.5 ± 1.8

BMI: body mass index. BP: Blood pressure. Ch: Cholesterol. LDL: Low density lipoprotein. HDL: High density lipoprotein. TG: Triglycerides. WC: waist circumference. HOMA-IR (homeostasis model assessment-insulin resistance). (*) p < 0.05, between groups.

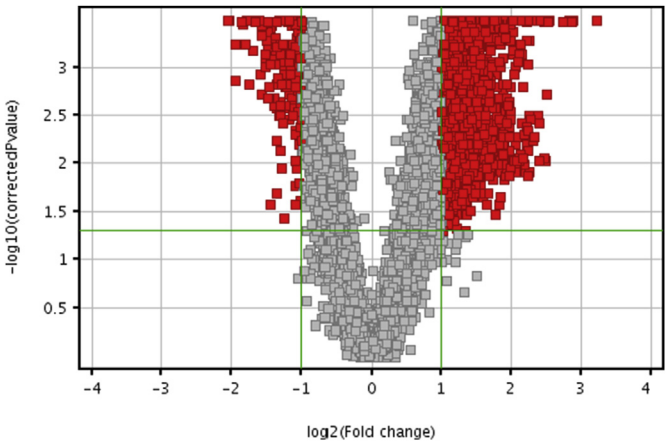

Fig. 1. Volcano plot analysis applied to the microarray data revealed 1436 genes that were significantly expressed (p < 0.01 with FC 1.5 (up or down)) in obese patients compared to controls. The plot show a log 2-fold change in mRNA expression between the 2 groups on the X-axis and the negative log of t-test p values on the Y-axis. Each gene was represented by a single point and dark gray areas indicate genes with significant changes in gene expression.

In the gene set of cellular function, the most relevant genes were C-terminal region of Nel-like molecule 1 protein (NELL1) and Pigment epithelium-derived factor (SPEDF), these genes were over expressed. In the gene set of metabolism, insulin growth factor type 1 (IGF1), ApoA5 (apolipoprotein subtype 5), Foxo4 (Forkhead transcription factor 4), ADIPOR1 (receptor of adiponectin type 1) and AQP7 (aquoporine channel protein 7) were over expressed. Moreover, PIKFYVE (PtdIns(3) P 5-kinase) and ROCK-2 (rho-kinase II) were under expressed.

4. Discussion

Our design of peripheral blood gene expression in obese and healthy subjects, by microarray shows that obesity features singular gene expression signatures. We included non obese subjects in these studies as an example of a status with a known peripheral blood gene expression profile and for purposes of contrasting the metabolic expression signatures to that of an obesity status without metabolic disorders. As we expected, gene expression of the obese subjects was enough to discriminate these patients from control.

In the gene expression set of obesity, a disease that represents more risk to cardiovascular disease and cancer, we see elevated expression of genes associated with activation, signaling and function of cells. Several of these cell activation genes are also related with T cell. The up-regulation of T cell activation seen in the literature may be a byproduct of enhanced activation of the immune response by adipocytes. Some studies have shown activated T cells to be present in elevated amounts in visceral adipose tissue of mice with obesity and diabetes mellitus type 2 [15,16]. The triad of obesity, metabolic syndrome, and diabetes mellitus type 2 are typically considered metabolic, not immune, diseases although aspects of each involve inflammation, sometimes systemically. However, metabolic syndrome and diabetes mellitus type 2 were characterized by a recognizable peripheral blood gene by expression discriminating each state from control [11]. In our study, we identify the differentially expressed genes in obese patients without metabolic complications, and as far as we know, is the first time in the literature [17]. The gene expression profile distinguish obesity consisted of many genes involved in cellular function and metabolism.

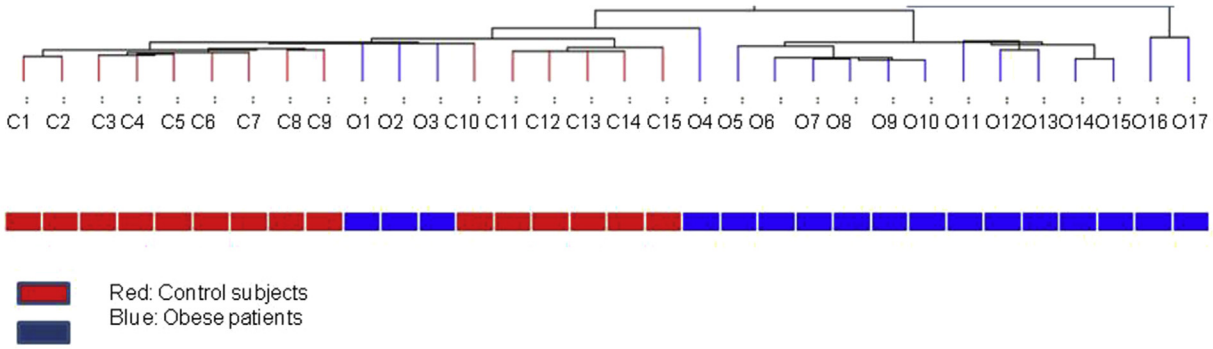

Fig. 2. Supervised hierarchical clustering of obese patients vs healthy subjects.

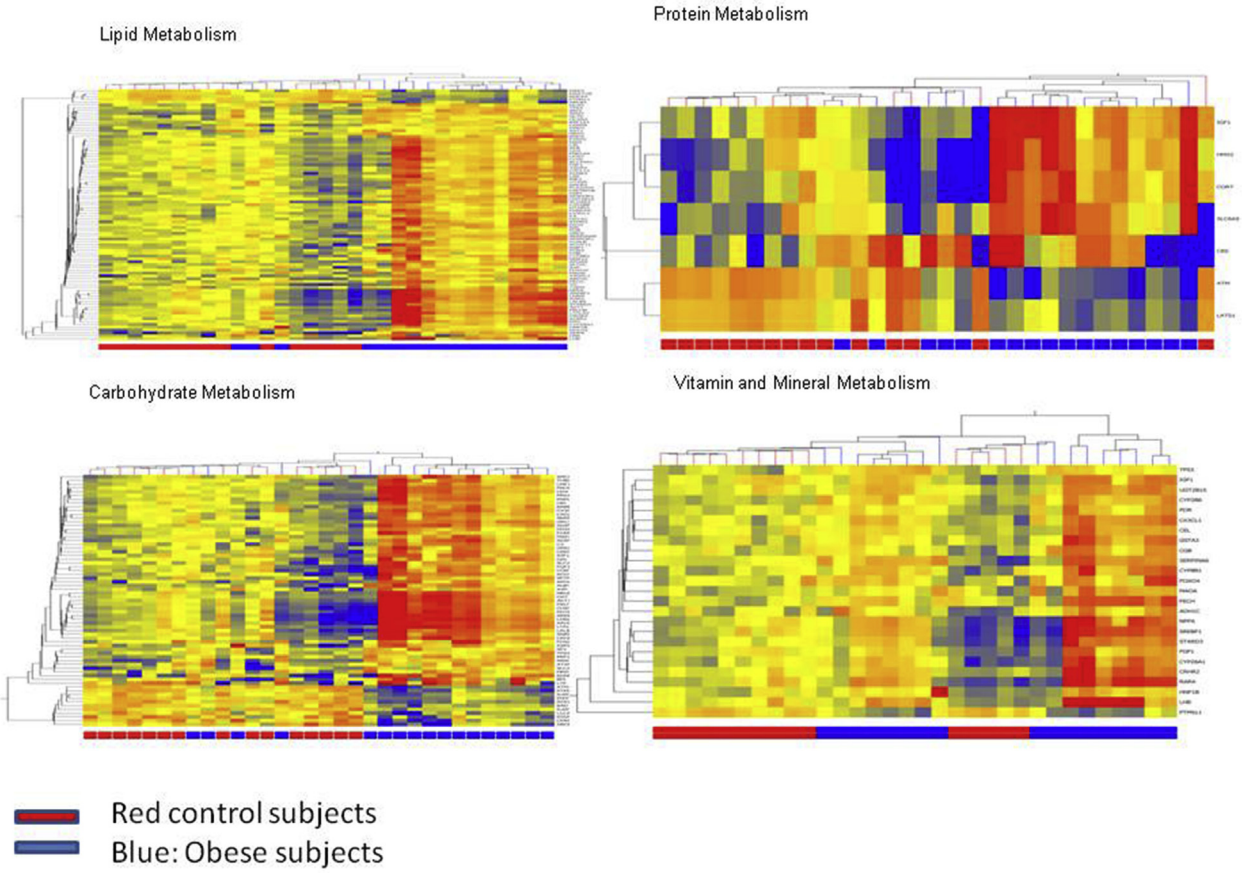

Fig. 3. Cluster analysis of category cellular function metabolism (red cluster as over expressed and blue as cluster under expressed). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

A potential source of typical gene expression among healthy subjects and obese patients was presence or absence of pharmacologic therapies. We performed this analysis in obese patients without drugs, thus showing that the pattern of expression is characteristic of patients with obesity and is not related to the effect of drugs.

Data from hierarchical clustering analysis realized on the list of 1436 differentially expressed genes showed two main biological process altered; cellular function and metabolism. From these two biological pathways, we obtained a list of 1248 differentially expressed genes (up-regulated) and 168 down-regulated in obese patients compared to the control group. Interestingly, we observed that highly up-regulated genes were involved in metabolism, insulin growth factor type 1 (IGF1), ApoA5 (apolipoprotein subtype 5), Foxo4 (Forkhead transcription factor 4), ADIPOR1 (receptor of adiponectin type 1) and AQP7 (aquoporine channel protein 7) and down-regulated; PIKFYVE (PtdIns(3) P 5-kinase) and RockII (Rho kinase type II). First, some lines of evidence had shown that APOA5 genetic variants are related with obesity and non-HDL-C in Chinese children and adolescent [18] and APOA5 is related with intracellular homeostasis in obese subjects, too [19]. Secondly, PIKFYVE is related with T cell and IFN gamma actions in inflammatory status, as is expected to be altered in the obesity status [20]. Thirdly, deletion of hepatic Fox1/34 genes in animal models impacts on

Table 2
Category cellular function and metabolism.

	p-value
Cellular function	
Cell morphology	2.22E-06–8.51E-03
Cell death and survival	5.79E-06–8.42E-03
Cellular development	1.63E-05–8.48E-03
Cellular growth and proliferation	1.63E-05–8.39E-03
Cell cycle	1.65E-03–8.03E-03
Cellular compromise	3.11E-03–7.55E-03
Cell-to-cell signaling and interaction	6.77E-05–8.42E-03
Cellular movement	2.83E-04–8.47E-03
Metabolism	
Carbohydrate metabolism	8.69E-04–7.01E-03
Lipid metabolism	4.96E-05–8.28E-03
Vitamin and mineral metabolism	3.44E-03–7.81E-03
Protein synthesis	4.06E-03–4.06E-03
Amino acid metabolism	5.37E-03–5.37E-03

p value: A measure of the likelihood that the association between a set of genes in your dataset and a related function is due to random association. The smaller the p-value, the less likely that the association is random and the more significant the association. Fischer test.

glucose metabolism through down regulation of gluconeogenesis and upregulation of glycolysis [21] and Foxo4 expression is under regulated in our obese subjects. Fourthly, Miranda et al. [22] have reported the evidence of coordinated relationship between adipose aquaglyceroporins, with a higher expression found in visceral fat, and between subcutaneous adipose AQP7 and hepatic AQP9 gene expression within morbid obesity.

Finally the upregulation of ADIPOR1 in obese subjects could be related with lipid metabolism and other metabolic disorders [23] and the down-regulation of RockII is important in obese subjects because the newly detected functions of ROCK in regulating glucose and energy metabolism, with an important emphasis on metabolic actions of insulin and some adipokines such as leptin [24].

The present study has an important strength; a control population of healthy subjects has been included. Some limitations of our study are; firstly, only 17 obese patients were enrolled due to the difficulty in finding newly diagnosed obese patients without metabolic complications and drug treatments which was one of our exclusion criteria. Similarly, it is noteworthy that other studies using microarray technology in metabolic disorders also have analyzed similar number of samples [25,26]. The lack of information in our design for the leucocytes and lymphocyte composition could be a potential bias in our study. Finally, many uncontrolled non-genetic factors such as exercise and diet could influence the relationships found with our design. Besides the limitations mentioned above, we showed that PBMCs from obese patients presented significant changes in gene expression, exhibiting 1436 differentially expressed genes compared to PBMCs from non-obese subjects.

An important concept of our work is that PBMC could be a representative measure for tissue changes. Liew et al. [10] showed that the comparison between blood expressed genes and nine tissue types revealed that a large proportion of genes expressed in the nine tissues are also expressed in blood cells. Moreover, there is a lack of studies in obesity area. Only one study have detected a high expression of visfatin mRNA in with omental adipose tissue and peripheral mononuclear cells, without correlation with BMI [27]. In a second study [28], authors showed a positive correlation between expression of adipokines in adipose tissue and peripheral mononuclear cells. Therefore, more studies of correlation between the expression of the adipose tissue genes and the peripheral mononuclear cells are necessary in this area of work, in order to obtain biomarkers with easy access in peripheral blood.

Furthermore, our data showed a higher number of genes involved in biological processes implicated in metabolism, with genes presenting high fold-change values (up-regulation and down regulation) associated with lipid, glucose and protein metabolism.

Conflict of interest

None.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.clnu.2017.06.006.

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